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TOXICITY OF MICRONUTRIENTS TOWARD CORN LEAF CATALASE

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Presently science is rather sure about the essentiality of 15 elements for plant growth. These 15 are: H, B, C, N, O, Mg, P, S, K, Ca, Mn, Fe, Cu, Zn, and Mo. Of these fifteen B, Mn, Fe, Cu, Zn, and Mo are required only in small amounts and are called micronutrient elements or trace elements. The others are used in comparatively large amounts and may be referred to as macronutrient elements. Iron was the first micronutrient element found to be essential to plant life, and the essentiality of the other five has been established only in recent years.

The literature contains some references which pertain to the effects of micronutrients on the relative activity of catalase. Rather than measure the toxic effects of any micronutrient element, these investigations in the literature more nearly determine the relative amount of catalase formed without the micronutrient element or with varying amounts of the micronutrient in the nutrient solutions. Bailey and McHargue (1944) working with tomato leaves found that maximum catalase activity occurred when 0.01 p.p.m. copper was added to the nutrient solutions, and that with higher concentrations of copper there was a decrease in catalase activity. They also reported that catalase activity in tomato fruits diminished progessively with additions of 0.01, 0.05 and 0.1 p.p.m. copper to the nutrient solutions, and that catalase activity in alfalfa leaves was at a maximum when 1 p.p.m. zinc was added to the nutrient solutions as compared to 0, 0.5 and 2 p.p.m.

The catalase activity of alfalfa was reported by Bailey and McHargue (1944) to be depressed by the addition of 1 and 2 p.p.m. manganese to the nutrient solutions. However, Pattanaik (1950) found that additions of manganese as high as 10 p.p.m. to rice plants growing in culture solutions increased the catalase activity, and it was postulated by him that the catalase activity was affected by the direct influence of manganese on the iron containing prosthetic group of the catalase molecule. There are also conflicting reports for the catalase activity in squash plants. Alexander (1942) found that the catalase activity in squash plants was increased by boron deficiency, whereas Bailey and McHargue (1944) reported that catalase activity in alfalfa was at a maximum with 1 p.p.m. boron as compared with 0.1, 0.25 and 0.5 p.p.m.

The general effect of inorganic ions on plant catalases was previously reviewed (Eyster, 1953).

METHODS AND MATERIALS

The method described previously (Eyster, 1953) was used for the quantitative determinations of catalase activity. A "dilution" method was used for copper and

zinc salts which were effective in such small concentrations that the amount could not be weighed and added directly to the macerated leaf suspension. In this "dilution" method, the same macerated sample was handled in the following sequence: (a) two catalase measurements reducing volume from 25 ml. to 21ml.,

TABLE 1

Toxicity of Micronutrients toward Activity of Catalase in Macerated Corn Leaves. Catalase Value is Expressed as Milliliters of O_2 in a 5-Minute Interval.

Micronutrient Compound	Concentration of Micronutrient Element	Catalase Activity* A Without Compound	Catalase Activity* B With Compound	B/A
	p.p.m.	ml.	ml.	%
$Na_2B_4O_7.10H_2O$	100 B	5.52	5.40	98
	500 B	11.88	11.54	97
	2000 B	12.10	11.06	91
	2500 B	11.84	<8.42	<71
$Na_2MoO_4.2H_2O$	100 Mo	6.54	6.41	98
	500 Mo	7.40	7.20	97
	2000 Mo	7.76	7.26	94
	3000 Mo	6.72	6.43	96
	5000 Mo	6.72	7.20	107
	15000 Mo	7.13	6.32	89
$MnCl_2.4H_2O$	75 Mn	8.24	8.04	98
	100 Mn	8.23	7.46	91
	100 Mn		0.24†	
	125 Mn	8.46	8.40	99
	200 Mn	8.21	7.38	90
	300 Mn	8.13	7.60	94
	2000 Mn	7.40	6.45	87
	2500 Mn	8.26	7.44	90
	3000 Mn	7.59	5.86	77
	3000 Mn		0.66†	
	3500 Mn	8.33	5.60	67
$KMnO_4$	100 Mn		12.26†	
	100 Mn	8.62	9.46	110
	200 Mn	9.50	11.80	124
$FeSO_4.7H_2O$	0.1 N‡	11.54	9.84	85
			3.25†	
$FeCl_3.6H_2O$	0.1 N‡	10.91	5.16	47
			6.04	
$ZnCl_2$	50 Zn	7.17	3.38	47

*Each value is an average of two successive measurements.

†Value for blank, using micronutrient compound alone.

‡The concentration here is expressed in normality instead of parts per million. 0.1 N $FeSO_4.7H_2O$ represents 2800 p.p.m. Fe, and 0.1 N $FeCl_3.6H_2O$ represents 1867 p.p.m. Fe.

(b) addition of 4 ml. of dissolved micronutrient to bring volume back to 25 ml., (c) two more catalase determinations, final volume 21 ml., (d) addition of 4 ml. of dissolved micronutrient, final volume 25 ml., (e) two more catalase determinations, final volume 21., (f) addition of 4 ml. of dissoved micronutrient, final volume 25 ml., (g) two more catalase determinations. The concentration of the micronutrient

varied in (b), (d), and (f), and the concentration was computed so that it made a known final concentration in the 25 ml., allowing in (d) and (f) for the amount previously added. As for example in the case of $ZnCl_2$, 4 ml. of 6.25 p.p.m. Zn as $ZnCl_2$ was added in (b) giving a final concentration of 1 p.p.m. Zn, 4 ml. of

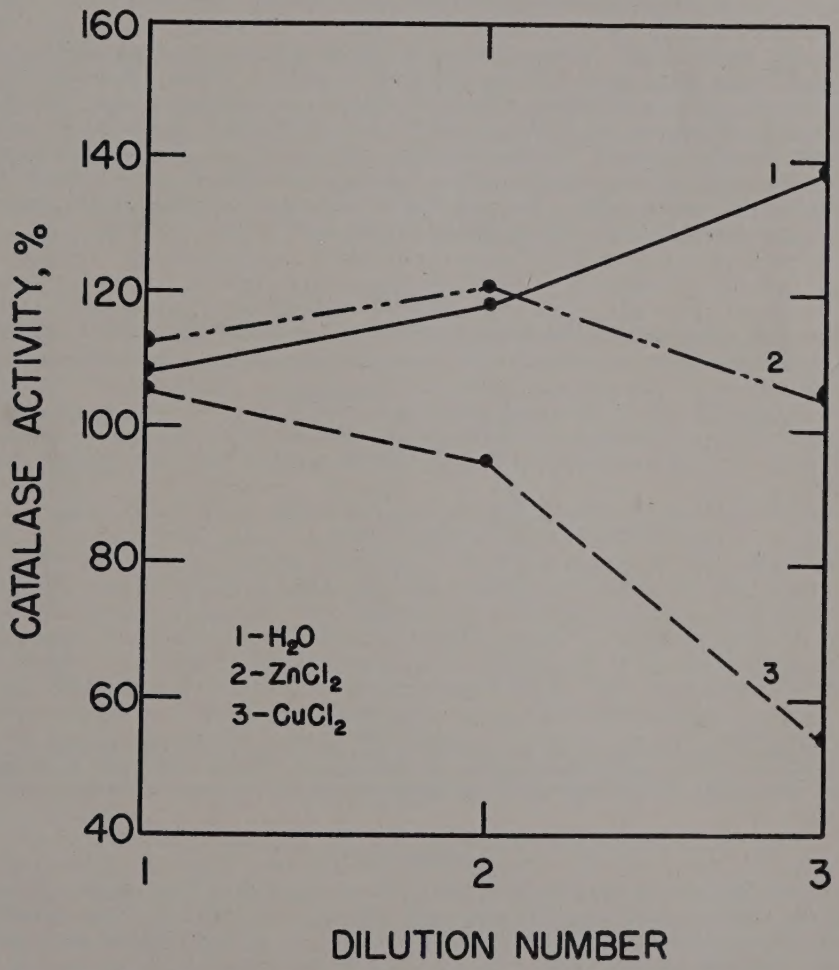

FIGURE 1. Toxicity effects of copper and zinc micronutrients toward catalase activity of macerated corn leaves determined by the "dilution" method. Concentrations: $ZnCl_2$—1 p.p.m. Zn, 10 p.p.m. Zn, and 50 p.p.m. Zn at dilutions 1, 2 and 3 respectively; $CuCl_2$—1 p.p.m. Cu, 10 p.p.m. Cu, and 50 p.p.m. Cu at dilutions 1, 2, and 3, respectively.

57.25 p.p.m. was added in (d) making 10 p.p.m. Zn, and 4 ml. of 260 p.p.m. was added in (f) making 50 p.p.m. Zn. It should be emphasized that concentrations refer to the pertinent element only and not the whole compound. The results were compared with a run in which 4 ml. of water was used for the additions in (b), (d), and (f).

RESULTS AND DISCUSSION

Toxicity effects of micronutrient compounds are recorded in tables 1 and 2. Only two micronutrient elements were found to have any marked degree of toxicity toward catalase activity. These were copper and zinc. The toxicity of copper chloride toward catalase activity became noticeable between 1–10 p.p.m. Cu, and that of zinc chloride between 10–50 p.p.m. Zn (fig. 1). The toxicity limits of the other micronutrient elements were found to be: about 2500 p.p.m. of Mn (0.041 M $MnCl_2.4H_2O$), about 2000 p.p.m. of B (0.043 M $Na_2B_4O_7.10H_2O$), and no toxicity at 5000 p.p.m. of Mo (0.052 M $Na_2MoO_4.2H_2O$). The toxicity of iron compounds was somewhat difficult to determine because of their "catalase" properties. However, 0.1 N $FeSO_4$ (2800 p.p.m. of Fe) still permitted 85 percent of full catalase activity. Measurements with ferric and ferrous compounds, as well as with permanganate and manganous compounds may indicate that catalase is extremely stable. The reducing environment of ferrous sulfate appeared to be more favorable than the oxidizing environment of ferric chloride.

TABLE 2

Toxicity of Copper and Zinc Compounds Toward Catalase Activity by the "Dilution" Method.

Compound	Dilution	Concentration of of Element	Catalase Activity†
		p.p.m.	%
Water*	1st		108
	2nd		118
	3rd		139
$CuCl_2$	1st	1 Cu	106
	2nd	10 Cu	96
	3rd	50 Cu	55
$ZnCl_2$	1st	1 Zn	113
	2nd	10 Zn	121
	3rd	50 Zn	106

*Control.

†Based on the average of two measurements, the milliliters of O_2 actually produced in a 5-minute interval divided by the expected and then expressed in per cent, where the expected O_2 production was calculated by assuming direct proportional decrease by dilution.

SUMMARY

Of the six micronutrients (Fe, Mn, B, Zn, Cu and Mo) only Cu and Zn compounds showed any marked toxicity toward catalase activity. The toxicity of copper chloride became noticeable between 1–10 p.p.m. Cu, and that of zinc chloride between 10–50 p.p.m. Zn.

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EXAMINATION OF TWO GOLD OBJECTS OF ABORIGINAL MANUFACTURE

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As a consequence of his publication in this journal of a paper (Caley, 1949) on the estimation of the fineness of gold objects by means of specific gravity measurements, the author has received a number of such objects for examination. Two of these objects seemed especially interesting as examples of aboriginal gold working, and the results of their examination are here presented. Both objects were submitted by Frederick R. Pleasants, Curator of Primitive Art, The Brooklyn Museum.

One of the objects, a large pendant apparently intended as a breast ornament, came from the Ivory Coast, West Africa, and probably was made sometime in the last century. Its general form is that of a low hollow cone with a diameter at the base of 9.5 cm. and a maximum height of 3.5 cm. It represents a coiled snake holding a frog or toad in its mouth. From directly above, the object appears to be solid, but only the top half of the body of the tightly coiled snake is actually present. Both the scales of the snake and the spots on its victim are conventionally, but artistically, represented. The shape of the object and the absence of any sign of hammering or tooling shows that it must have been cast in one piece by the *cire perdue* method. Its production by casting is further indicated by the traces of carbonized molding sand still present in some of the crevices about the mouth of the snake.

The entire surface of the object has the rich yellow color of gold of high fineness, except for a few minute areas on metal in higher relief on the bottom of the rim of the base where slight wear has occurred. Here the surface is very pale yellow. By making test scrapings in a few inconspicuous places it was seen that the metal below the surface is generally very pale in color, almost like silver in appearance. The weight of the object was found to be 83.49g., and its specific gravity at 25°C was found to be 12.05. Chemical tests on minute specks of the underlying metal showed that the alloy contains both silver and copper, with the latter in slightly higher proportion. The average gold content of this object as derived from its specific gravity and these tests is only about 40 percent, or in other words the metal as a whole is only about 400 fine or about 9.6 karat. Native gold of this composition is very rare, and it therefore is very probable that the alloy was especially prepared.

This object in all probability was colored by the *mise en couleur* process. That is, the original casting of base gold was first treated with some corrosive paste or liquid to dissolve out the baser metals from the surface, leaving pure gold in a finely divided state on the surface. Bowditch, an eye witness of the methods used by native gold workers among the Ashanti of West Africa, in 1819, reports that, "To give the gold its proper color, they put a layer of finely ground red ochre (which they call inchuma) all over it, and immerse it in boiling water mixed with the same substance and a little salt; after it has boiled half an hour, it is taken out and thoroughly cleansed." This account is perhaps incomplete, as red ochre and salt water would not be effective in removing base metals from the surface rapidly, though the mixture would be effective for polishing to a clean, bright surface. Possibly some acid vegetable extract was also added, for Zeltner reports that water, salt, and brew from the tamarind fruit was used by the Bambara for cleaning copper castings. Regardless of the way in which the baser metals were removed from the surface, it is unlikely that the color could have been developed only by polishing or

burnishing, for the attaining of a fine gold color after corrosion is very difficult by such means when the alloy is less than 500 fine. It is much more likely that the object was first heated, perhaps with a blowpipe, to fuse the gold on the surface, prior to any final cleaning and polishing. There is no indication of the application of gold leaf or of gilding by amalgamation, and there would be no reason for using such processes when the metal was a gold alloy. The whole method of making this object is indicative of a high state of skill and practice in the art of gold working.

The other object, a plain, hemispherical bowl, came from Colombia, South America, and was said to have been produced by hammering out a single large gold nugget. The date of its manufacture is unknown. Its maximum diameter at the rim is 11.2 cm., its minimum diameter is 10.7 cm., and its height is 5.3 cm. The average thickness of the metal as measured at the rim is 0.15 cm. Both the inside and outside surfaces of the bowl are covered with small hammer marks. A few cracks and indications of closed cracks and folds are evident on the outside surface, especially at the bottom.

The color of the metal is decidedly lighter than that of fine gold, and is similar to that of brass. Test scrapings on the rim showed no indications of any enrichment of the surface. The weight of the object was found to be 227.8 g., and its specific gravity at 25°C was found to be 16.13. Chemical tests on minute scrapings from the rim showed that the metal was composed mostly of gold and silver with only a small proportion of copper, which was estimated to be about 1 percent of the whole. The gold content of the metal as dervied from both its specific gravity and these tests is about 77 percent, which corresponds to 770 fine or 18.5 karats.

According to Rivet and Arsandaux (1946) native gold from Colombia is mostly argentiferous and contains little copper. In their list of assays of 56 specimens of native gold from Antioquia, the principal gold-producing province, the range of gold content is from 63.4 to 96.5 percent with an average of 82.6 percent. The gold content of specimens from certain sites is almost the same as that of the object here discussed. Assays of native gold from other provinces show a range from 73.5 to 98.0 percent, with an average of 85.3 percent. These authors also list quantitative analyses of 23 objects said to have been made from Colombian native gold. The gold content of these objects ranges from 68.3 to 91.4 percent, the silver content from 8.6 to 31.7 percent, and the copper content from 0.0 to 1.9 percent.

Both the appearance of this bowl and its chemical composition are therefore consistent with the report that it was made by hammering out a nugget of Colombian native gold. In contrast with the first object, this second one was made by the most primitive method of gold working.

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The story of Ohio's mineral resources is well illustrated with maps and photographs of the industries developing and utilizing the various mineral resources of the state. F. W. F.

PROTEASE STIMULATION BY FOODS IN ADULT *AEDES AEGYPTI* LINN.[1]

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Much time and money have been devoted to the study of malaria and its vectors. However, most of the research and financial support for it has gone into fields other than the physiology of the various parasites, especially pertaining to their infectivity of mosquitoes. According to Huff (1941 a), "Hardly any other phase of malariology has been so badly neglected as the study of all factors influencing infection in the mosquito."

Of the relatively small amount of data dealing with the malarial parasite infectivity in mosquitoes, a large part of it deals with studies of strains of mosquitoes resistant or susceptible to strains of malarial parasites.

This particular study was undertaken to extend the small field of knowledge of effect of various foods on digestive enzyme elaboration. Since the work of Schlottke (1937 a, b, c), there has been only the work of Day and Powning (1949), Fisk (1950), and Fisk and Shambaugh (1952) studying the stimulatory effects of food on enzyme elaboration. Such research serves to fill in many gaps in the knowledge of the physiology in adult mosquitoes, a physiology not well understood yet extremely important in all considerations of malarial infectivity in mosquitoes.

METHODS

Aedes aegypti Linn. were selected for this study because of the relative ease with which great numbers can be reared. They were obtained from the Research Foundation Project in the Department of Zoology and Entomology sponsored by the United States Public Health Service, which maintains an active colony for its own use.

Female mosquitoes four to six days old were used, for they feed most readily at that age and by that time have disposed of any larval food which remained in the digestive tract at emergence (Fisk, 1950). Four to six days after emergence the mosquitoes were placed in a lamp chimney covered at the top with cheesecloth.

Feeding and Dissection Techniques. Mosquitoes were fed either from the bare forearm placed on the cheesecloth on the top of the lamp chimney or through an artificial membrane in a heated feeding apparatus to be described later. The mosquitoes were attracted to the arm or warmed membrane and fed through the gauze. After each one had engorged partially with blood or a blood substitute solution, she was withdrawn from below with an aspirator which was inserted into the chimney through an opening in a cross-slitted rubber dental dam stretched over an embroidery hoop. Each female was placed in a separate vial which was stoppered with a wad of dampened cotton. The fed mosquitoes were placed in an incubator at 30° C. After 18 hr. for digestion, the midguts were dissected from the mosquitoes by the technique outlined by Fisk and Shambaugh (1952).

Individual Mosquito Weighing. In this laboratory, as similarly reported by Roy (1936), the range of weight of mosquitoes at a constant age is greater than the average weight for an individual. In order to determine accurately the amount

[1]From a dissertation submitted to the Faculty of the Graduate School of The Ohio State University in partial fulfillment of requirements for the degree of Doctor of Philosophy.

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of blood ingested by a female mosquito during feeding, it was necessary to obtain individual weights.

A small, rectangular, plastic container was constructed (3.6 cc.) to hold the mosquitoes while weighing them on a Roller-Smith Precision Balance (spring torsion). The mosquitoes were anesthetized with carbon dioxide before weighing. With an aspirator they were transferred to marked shell vials and after revival were allowed to feed on the bare forearm. The amount of blood fed was regulated by disturbing the insect during feeding. Each mosquito was reweighed soon after feeding in the manner just described. The mosquitoes were returned to the marked vials which were stoppered with a wad of damp cotton and placed in an incubator at 30° C for eighteen hours.

Artificial Feeding Apparatus. It was necessary to construct an apparatus to keep artificial blood solutions at a constant temperature, 40° C. An "oven" was constructed as follows. Two sizes of Lucite plastic tubing were used: 1) outside diameter 4 cm., inside diameter 3.5 cm., length 3 cm. and 2) outside diameter 5 cm., inside diameter 4.4 cm., length 5 cm. The edges of the tubing were ground flat and smooth with powdered carborundum and alumina. The smaller tube was covered at one end with Baudruche Membrane held in place with a rubber band. When inverted this acted as a reservoir for the solutions. Nichrome resistance wire was wound around the outside of the larger tube and soldered to regular 110 volt copper extension wire. The wires were held in place with an outer insulating layer of furnace cement about one centimeter thick. The smaller tube containing the solutions was placed within the larger tube. This apparatus is a modification of that described by Greenberg (1949).

A cover was prepared for the larger tube to prevent excessive heat loss. A thin, circular, plastic disc 6.3 cm. in diameter was joined with acetone to a plastic ring 4.3 cm. in diameter and 1.5 cm. wide. The heating element was joined in series with a variable rheostat and a 100 watt incandescent bulb. It was necessary to vary the rheostat manually since there was no thermostat in the circuit.

The assembled feeding apparatus was placed on the cheesecloth on the top of the lamp chimney containing mosquitoes. The capping membrane of the inner tube, wet with distilled water, rested on the cheesecloth. Accurate readings of the temperature at the membrane surface were taken with a Leeds and Northrup single range potentiometer connected to a copper-constantan thermocouple placed between the cheesecloth and the capping membrane.

Preparation of Blood Substitute Solutions. In *Aedes aegypti,* ingested blood is taken directly into the midgut or rarely the diverticula. Sugar solutions without erythrocytes, in practically all instances, were taken into the diverticula (Trembley, 1952). Most protease activity occurs in the midgut after feeding (Fisk and Shambaugh, 1952). In view of these facts, it was decided to use sheep erythrocytes with substitute solutions so that solutions would be primarily dispatched to the midgut. Fresh sheep blood was defibrinated by mixing 10 percent of its volume with 3 percent sodium citrate. Whole sheep blood was centifuged 10 min. at 5500 rpm. The supernatent serum was siphoned off. The remaining erythrocytes were diluted to the original volume with physiological saline (0.85% sodium chloride) and stirred before recentrifuging. The washing procedure was repeated twice more. The subsequent thrice-washed erythrocytes were used in all tests requiring erythrocytes.

It was decided to test for any response in protease activity to various fractions of whole blood and to plasma protein fractions. The following solutions were offered: whole human blood, whole sheep blood, washed erythrocytes (cells)+saline, erythrocytes+gum acacia, erythrocytes+serum dialyzate, erythrocytes+serum proteins (non-dialyzable), erythrocytes+0.6 percent fibrinogen, erythrocytes+0.6 percent albumin, erythrocytes+0.6 percent gamma-globulin. The plasma protein fractions were bovine. All of these mixtures were used in a 1:1 volume ratio,

except the solution of erythrocytes+albumin+gamma-globulin+fibrinogen which was in the ratio of 3:1:1:1.

Several techniques were tried to obtain a protein-free plasma but the most useful was dialysis. Ten milliliters of sheep blood were placed in Visking tubing. It was dialzyed in 30 ml. phosphate buffer, pH 7.8, for 3 hr. in an Aminco Mechanical Dialyzer. The dialyzate gave a negative test for proteins upon the addition of trichloroacetic acid. Plasma protein fractions were dissolved in distilled water. The solutions of fibrinogen contained between 40 and 50 percent sodium citrate to prevent its coagulation upon the addition of erythrocytes.

Injection Technique. A series of unfed mosquitoes was injected with blood from from 18-hour fed mosquitoes mixed with saline. The injection technique recently described (Shambaugh, 1952) was modified by injecting the saline into the fed mosquito just prior to dissection eighteen hours after feeding. In four instances the midgut contents were mixed with saline and injected into unfed mosquitoes.

Enzyme Analyses. The theory and technique for enzyme analyses of midgut breis are discussed adequately in a previous article (Fisk and Shambaugh, 1952). This procedure was used for protease determinations of dissected midguts in lots of 10 or 20. A modified small sample technique was used for less than 10 midguts in a dissection. One millilitier of buffer was added to the homogenate of midguts and the total was brought up to 2 ml. with mosquito saline. The remainder of the technique was unmodified.

RESULTS

The conclusions of Fisk and Shambaugh (1952) on the stimulation of proteolytic digestive enzymes after a blood meal as opposed to a meal on a sugar solution served as a basis for further studies of the effect of various blood fractions on protease elaboration in the midgut. The amount of protease activity

TABLE 1

Summary of data on protease activity of Aedes aegypti females eighteen hours after feeding on various food materials.

FOOD MATERIAL	AVERAGE ACTIVITY PER MIDGUT (Optical Density)
Starved	0.049
Cells + saline	0.144
Cells + 6% gum acacia	0.120
Cells + serum dialyzate	0.216
Cells + serum proteins	0.598
Cells + 0.6% fibrinogen	0.217
Cells + 0.6% albumin	0.256
Cells + 0.6% gamma-globulin	0.338
Cells + albumin + fibrinogen + gamma-globulin	0.377
Whole human blood	0.503

determined was the amount of available protease at the time of dissection, minus the losses due to technique. The amount or condition of the blood meal within the midgut at the time of dissection had no effect on this technique, for the colored materials would be equal in both blanks and runs while the blood protein would be removed by the trichloroacetic acid precipitation.

In the following tables and figures, protease activity will be expressed in terms of optical density of the final solution described in the previous section. Optical density is a direct function of protease activity, being derived from the readings of

the colorimeter by multiplying them by the constant 0.002 (Hawk, et al., 1947). In these tables the number of midguts in the final solution read in the colorimeter depends on the initial number of insects dissected and the technique followed.

Results With Various Blood Fractions. Since the peak of digestive enzyme activity was shown by Fisk and Shambaugh (1952) to be 18 hr. after feeding, it was decided to run the experiments under these conditions (see table 1). Starved, or unfed individuals, were used as a check. Thrice-washed erythrocytes were

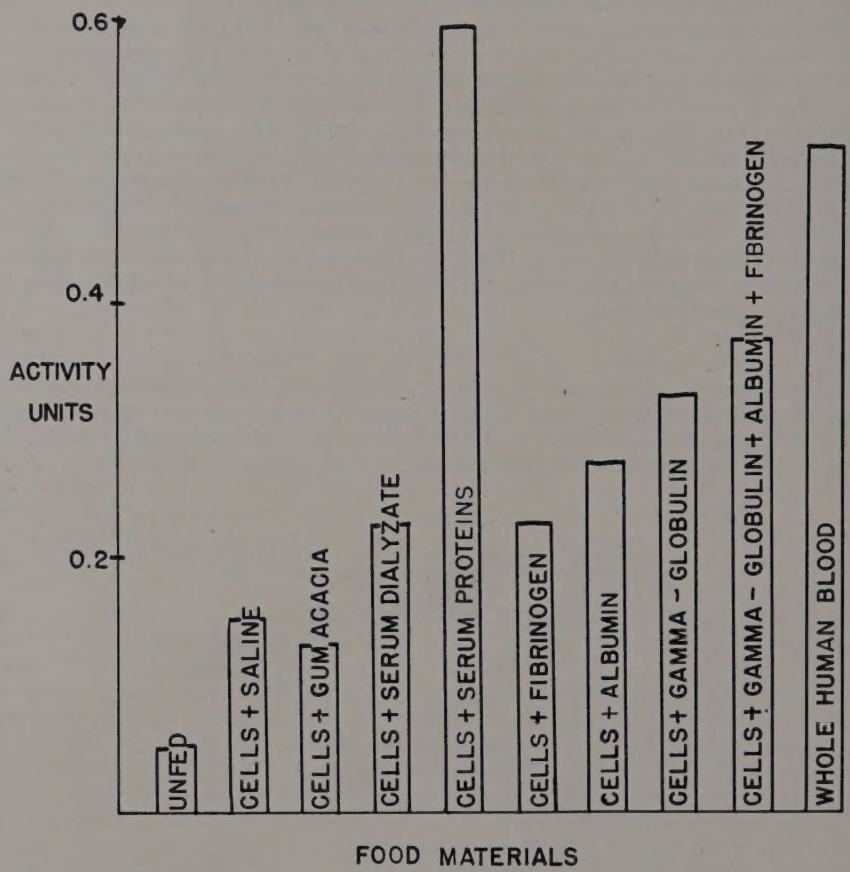

FIGURE 1. Relative amounts of protease activity in adult *Aedes aegypti* females in response to various food materials eighteen hours after feeding.

suspended in physiological saline to find the enhancement due to the cellular proteins in an inert medium. There was a slight increase in activity following the addition of the cellular proteins. Gum acacia was mixed with erythrocytes and was fed to discover any stimulatory effect due to viscosity or midgut distention. There was none. The addition of the prepared non-proteinaceous serum dialyzate increased the protease activity one and one-half times. When non-dialyzable sheep serum proteins were fed, a significantly higher amount of activity was demonstrated than with whole human blood. Both were significantly higher than the other materials tested here.

Three bovine plasma protein fractions (albumin, fibrinogen, gamma-globulin) were fed separately in 0.6 percent solutions. Protease activity with fibrinogen was not different from that with serum dialyzate. Activity with albumin was slightly higher, whereas gamma-globulin increased enzyme activity substantially. Additivity in stimulatory effect by the plasma fractions was tested by feeding the mixture of three fractions with erythrocytes. Enzymatic response to this mixture was greater than to any one fraction, but was not greater than the summed effect of the separate fractions (in excess of the value for erythrocytes alone). This is shown in figure 1.

Relation of Activity to Amount of Blood Ingested. Table 2 shows the quantitative effect of human blood on protease activity. The weight of blood per midgut is calculated from the weight of ingested blood divided by the number of mosquitoes fed that amount and not by the number of midguts in the final solution. These data were analyzed by the bivariate correlation coefficient test. The calculated

TABLE 2

The effect of the weight of whole blood on the protease activity in adult Aedes aegypti females.

Wt. Blood Ingested (mg.)	Protease Activity (Opt. Dens.)	No. of Midguts*	Activity Per Midgut	Wt. Blood Per Midgut	Wt. Blood Per Activity Unit (Opt. Dens.)
1.10	0.044	0.77	0.057	0.28	4.9
2.18	0.050	0.77	0.065	0.55	8.5
2.85	0.148	0.77	0.192	0.71	3.7
3.43	0.205	0.77	0.267	0.86	3.2
4.75	0.272	0.77	0.353	1.19	3.4
6.93	0.385	0.96	0.401	1.38	3.4
7.01	0.453	1.73	0.262	0.77	2.9
7.25	0.417	0.77	0.542	1.81	3.3
8.01	0.389	0.77	0.505	2.01	4.0
8.30	0.500	1.35	0.370	1.18	3.2
8.31	0.399	0.77	0.518	2.08	4.0
9.64	0.490	0.77	0.636	2.41	3.8
10.95	0.417	0.58	0.718	3.65	5.1
11.03	0.466	0.77	0.605	2.76	4.6

*Number of midguts in the final solution.

correlation coefficient of 0.944 indicates a strong positive interaction between the weight of blood ingested by a mosquito and its subsequent protease activity. This rectilinear relationship is shown in figure 2. This interaction remains whether a small number of mosquitoes was fed large volumes of blood or a large number of mosquitoes was fed small amounts of blood.

A set of three tests was made to test for the presence of a possible zymogen-kinase relationship in these digestive enzymes. Nine mosquitoes were fed normally on the forearm and allowed to digest the meal for 18 hr. The amount of blood ingested was determined. A similar amount of blood was mixed with nine freshly homogenized midguts and incubated 18 hr. at 40° C while the same amount of blood was incubated and then mixed with nine freshly homogenized midguts. The modified small sample technique for enzyme analysis was run on these three tests. There was no significant difference between the boiled blanks and runs in any test except for the normally fed mosquitoes, indicating that no zymogen-kinase reaction exists *in vitro*.

Results of Injected Series. Unfed mosquitoes injected with a mixture of saline and insect blood from blood-fed mosquitoes showed no increase in enzyme activity 8 or 10 hr. after injection (optical density of the final solution, 0.014).

Enzyme analyses were not possible on unfed mosquitoes injected with a mixture saline and midgut contents of blood-fed mosquitoes since three to four hours after injection, many of the internal viscera including the midgut were digested, leaving the tracheae seemingly intact.

Bases for Calculation of Dilution Factors and Activity Units. Assuming the average volume and the average weight of midgut tissue per female if 0.03 cu. mm. and 0.03 mg., respectively (Fisk, 1950), the milligrams of female midgut tissue per milliliter and the ratio of the volume of midgut tissue to the final solution read in the colorimeter can be calculated. In the case of 20 midguts, these figures would be 0.0132 mg. and 1:20,831, respectively. More detailed computations are given by Shambaugh (1953).

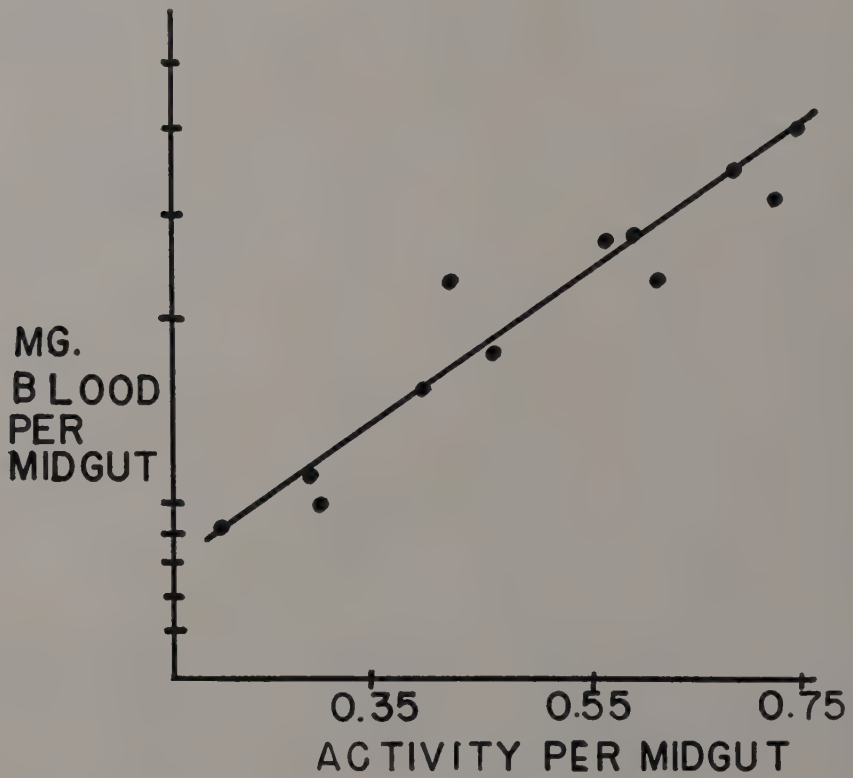

FIGURE 2. The ratio of the amount of blood ingested to the protease activity in adult *Aedes aegypti* females.

Protease activity may be expressed in terms of the velocity constant, K, of enzyme reactions:

$$K = \frac{1}{t} \times 2.3 \log. \frac{C_1}{C_2}$$

where C_1 and C_2 are the initial and final concentrations of protein substrate respectively after t minutes of digestion. The ratio of substrate concentrations can be derived from the optical densities as previously stated.

The initial substrate concentration (C_1) would correspond to a 1:3 dilution of the stock solution of the azoprotein substrate. This calculated figure is 7.36.

The final substrate concentration (C_2) is the difference between C_1 and the optical density of each trichloroacetic acid filtrate. Enzyme activity of the original midgut solution may be obtained by multiplying the velocity constant by the total dilution factor of each solution (Charney and Tomarelli, 1947). The activity units (actually velocity constants) are arbitrary and are not to be confused with the Willstäter units for trypsin activity. In spite of the calculations required, these units have no more merit than the optical densities used to express protein concentration and thus protease activity. Therefore they have not been calculated here, but further examples are given by Shambaugh (1953).

DISCUSSION

The mere presence of an enzyme in the digestive tract of an insect does not irrevocably establish its substrate as a food of the particular insect (Fraenkel, 1940), although in insects the correlation between food and enzymes to digest them is good. Perhaps the correlation of these enzymes with their actual substrates can be made more evident by a more thorough research program on enzyme specificity and activators. Yet MacGregor (1931) reported that male mosquitoes, force-fed by the "capillary-tube" technique of unsheathing the fascicle, were capable of digesting blood, normally never taken by males, within the same period of time required by females. This technique was tried, but the author was unsuccessful in his attempt to induce either sex of mosquitoes to ingest blood in this manner.

Although Day and Powning (1949) observed that all enzymes were stimulated irrespective of the food given to American and German roaches (omniverous feeders), preliminary results with an intermittent feeder on a restricted diet, such as the female *Aedes aegypti* mosquito, indicate a differential stimulation of enzymes dependent on the food offered these insects (Fisk and Shambaugh, 1952; Fisk and Shambaugh, in press).

The mechanism responsible for stimulation may be either hormonal or secretogogue (due to food or food products). Histological evidence of an increase in regenerative cells in the midgut of *Tenebrio molitor* after the injection of blood from fed beetles into unfed beetles led to the conclusion that the enzyme stimulation in this species is hormonal (Day and Powning, 1949). Previous work and results in the above section indicate a secretogogue stimulation in *Aedes aegypti*.

To test for conceivable precursors or zymogens present in the midgut upon which an activator or kinase possibly present in the blood could act, unfed individuals were tested against blood-fed individuals as described in the previous section. The results indicate that no zymogen-kinase relationship with blood exists. Fisk (1950) reported that no combination of crops, salivary glands, and empty midguts showed any measurable increase in protease activity. However, his tests were run after only two hours incubation.

Since there was no evidence for activation by endocrine inducement nor for a zymogen-kinase relationship, one can consider the effect of the various fractions of blood on protease activity as given in table 1. The residual value is given for unfed or blood-starved individuals. The effect of cellular proteins in an inert medium (erythrocytes in saline) is somewhat greater than the residual value. Both the dialyzable and non-dialyzable portions of blood were tested. The dialyzable portion mixed with erythrocytes was one and one-half times as effective in enzyme stimulation as the mixture of erythrocytes in saline. This would indicate a dialyzable, weak, stimulatory factor in the plasma of blood. The non-dialyzable plasma proteins and erythrocytes resuspended in saline intensified the protease activity to a level four times that found for erythrocytes and saline, indicating a strong, stimulatory effect. There was no difference between the digestive enzyme activities of those mosquitoes fed whole human blood and those fed whole sheep blood.

Inasmuch as the non-dialyzable (mostly protein) fraction of the plasma stimulated the most activity, various fractions of plasma proteins were fed separately and, once, collectively. Fibrinogen excited an elaboration of enzymes comparable to that of the plasma dialyzate. Plasma albumin increased the enzyme activity more than fibrinogen but not as much as gamma-globulin. The mixture of the three 0.6 percent solutions of proteins and erythrocytes stimulated more activity than any of the fractions alone. However, this amount of activity was less than that of the sum of the differences of the enzyme activities of each protein fraction minus the value for cellular proteins. This discrepancy is probably due to an error in sampling (only one test was possible), or is derivable from the effect of having the concentration of proteins in the composite substitute solution different than in the separate solution.

There exists a strong interaction between the amount of blood ingested by female mosquitoes and subsequent protease activity. The calculated correlation coefficient was 0.944, indicating that the blood ingested serves to increase the amount of protein substrate for the midgut proteases.

In the past two decades much valuable information has been accumulated for the understanding of the physiology and infectivity of malarial parasites through the use of bird malarial parasites and culicine mosquites. MacGregor (1931) reported that avian malarial parasites failed to develop if ingested into the diverticula. This fact would suggest that some reaction in the midgut, perhaps some process of digestion, was necessary for development. Huff (1927, 1934) concluded that immunity was not explained by differences in digestion processes between resistant and susceptible strains of mosquitoes discernible by histological methods. Other than genetic variation, the degree of infectivity and oöcyst development are greatly influenced by temperature and not by humidity nor activity nor age of the mosquito (Huff, 1941 a, b).

The number of oöcysts developed in the mosquito midgut is not directly correlated to the number of infective cells in the meal. This would indicate factors limiting infectivity other than exposure. In an infected mosquito the greater the number of oöcysts, the greater the number of eggs laid, but an increase in the number of eggs laid does not necessarily increase oöcyst formation. This was interpreted to be correlated with the absorption of blood after digestion (Hovanitz, 1947). Eyles (1952a) was able to demonstrate that serum alone was sufficient for full development of the parasite, but one-quarter development could occur on erythrocytes in saline. The dialyzable portion of the plasma had no effect. Bovine plasma albumin promoted mosquito infection in concentrations less than six percent when compared with physiological saline alone, but infections were less intense than those resulting from defibrinated blood.

There are still undetermined factors in the vertebrate blood affecting infection. Transfusion of normal blood into highly parasitized chickens was found to enhance the infectiousness of the blood in two or three attempts. Size of oöcysts, as well as their number, was found to be affected by the intensity of infection in the chicken (Cantrell and Jordan, 1946; Eyles, 1952 b).

Physiological studies on the blood of malaria-infected persons show increases in the blood of cellular proteins (after cellular disintegration), fibrinogen, and euglobulin, with an accompanying decrease in plasma albumin. Since plasma proteins have been shown to affect both protease stimulation and malarial infectivity, a strong interaction between these processes, formerly discounted, is suggested.

SUMMARY

1. Results of previous studies which indicated that the stimulation of proteolytic enzyme in the midgut of *Aedes aegypti* is secretogogue in nature served as the basis for this research. The technique of feeding adult female mosquitoes blood substitute solutions through warmed membranes is described.

2. A series of mosquito injection tests supports the previously proposed secretogogue theory of protease stimulation. Tests for conceivable zymogens present in the midgut stimulated by a kinase carried in the ingested blood (similar to the trypsinogen-enterokinase relationship of vertebrates) were negative. Various blood fractions were tested for secretogogue influences. In order for blood substitute solutions to be dispatched primarily to the midgut of this species of mosquito where protease elaboration is confined, it was expedient to mix them with thrice-washed erythrocytes.

3. A residual value of protease activity is given for unfed, or blood-starved individuals. The effect of cellular proteins in a relatively inert medium (sheep erythrocytes in physiological saline) is somewhat greater than the residual value. Both the dialyzable and non-dialyzable portions of blood were tested. The dialyzable portion mixed with erythrocytes was one and one-half times as effective in enzyme stimulation as the mixture of erythrocytes in saline. This would indicate a dialyzable stimulatory factor in blood plasma. However, the non-dialyzable plasma proteins and erythrocytes resuspended in saline intensified enzyme activity to an amount four times that of erythrocytes in saline. Evidently the more imporant stimulatory factors are in the non-dialyzable plasma proteins.

4. The effect of three plasma protein fractions at a concentration of 0.6 percent was tested. Fraction I (fibrinogen), normally 6 percent of plasma proteins, stimulated a secretion of enzymes comparable to that of the plasma dialyzate. Fraction V (albumin), normally 48 percent of plasma proteins, provided an increase in activity above that of fibrinogen. Fraction II (gamma-globulin) normally *6* percent of plasma proteins, stimulated a secretion greater than the sum of the fractions I and V. Enzymatic response to the mixture of these three plasma proteins was greater than to any one fraction, but was not greater than the summed effect of the separate fractions (in excess of the value for erythrocytes alone).

5. Through a series of experiments utilizing individual weights it was possible to discover a positive correlation between the amount of blood ingested by female mosquitoes and the subsequent protease activity of their midguts. This would lead to the conclusion that an increase in ingested blood increases the amount of the protein for the midgut proteases.

6. Discussions of the effect of blood and blood fractions fed to mosquitoes upon the degree of infectivity with malarial parasites are given. Since plasma proteins have been shown to affect both protease stimulation and malarial infectivity, a strong interaction between these processes, formerly discounted, is suggested.

ACKNOWLEDGEMENTS

The writer is indebted particularly to Dr. Frank W. Fisk for his painstaking guidance and criticisms of this research and manuscript.

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Plant Diseases in Orchard, Nursery and Garden Crops. *Ernst Gram* and *Anna Weber.* Edited and adapted by *R. W. G. Dennis.* Translated from the Danish by *Evelyn Ramsden.* Philosophical Library, New York. 1953. 618 pp. $18.50.

This is a British translation of the second Danish edition of 1944 which was published at a time of acute need for use by farmers and orchardists, as well as by agricultural advisors. Although directed toward a practical-minded audience, the entire work was masterfully conceived, and the fundamental discussions of the nature of disease, and of the basis for control measures are excellent.

The body of the volume consists of descriptions and illustrations of disease symptoms of tree and bush fruit; vegetables and herbaceous fruit; and ornamental plants and trees. Useful diagnostic keys to the diseases of principal crops are included. Of special merit is the realistic inclusion of major deficiency diseases with specific recommendations for remedies. It is unfortunate that diseases incited by nemas were not similarly included.

Although successful in Denmark and England as a guide for practical controls, the volume will not have the same use in the United States. Many major diseases in the United States are omitted or only briefly discussed. There are many nomenclatural differences, and specific recommendations based on European proprietary compounds are inadequate without knowledge of the specific chemical compounds and concentrations in such preparations. Advances in fungicides since 1944 are virtually omitted.

Except for the high cost, the book would be recommended for all plant pathologists. Surely it should be made available in institutional libraries.

PATRICIA ALLISON.

THE TRANSFER OF RADIOIODINE ACROSS PLACENTA AND BREAST

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It is common knowledge that both the fetus and the nursing young receive their nourishment from the mother; the one by way of the placenta, and the other by way of the breast. Part I of this investigation, deals with the passage of iodine from mother to fetus and is largely in the nature of a review, while Part II, which pertains to the passage of iodine back and forth across the placenta is, to my knowledge, original work.

The first experimental evidence for the transfer of known substances across the placental barrier from mother to fetus was published by Mayer (1817) who gave indigo and safranin to the pregnant rabbit. Subsequently a great deal of investigative work has been done, and Nicoloux (1909) presented in his excellent review the most important researches in this field prior to 1909.

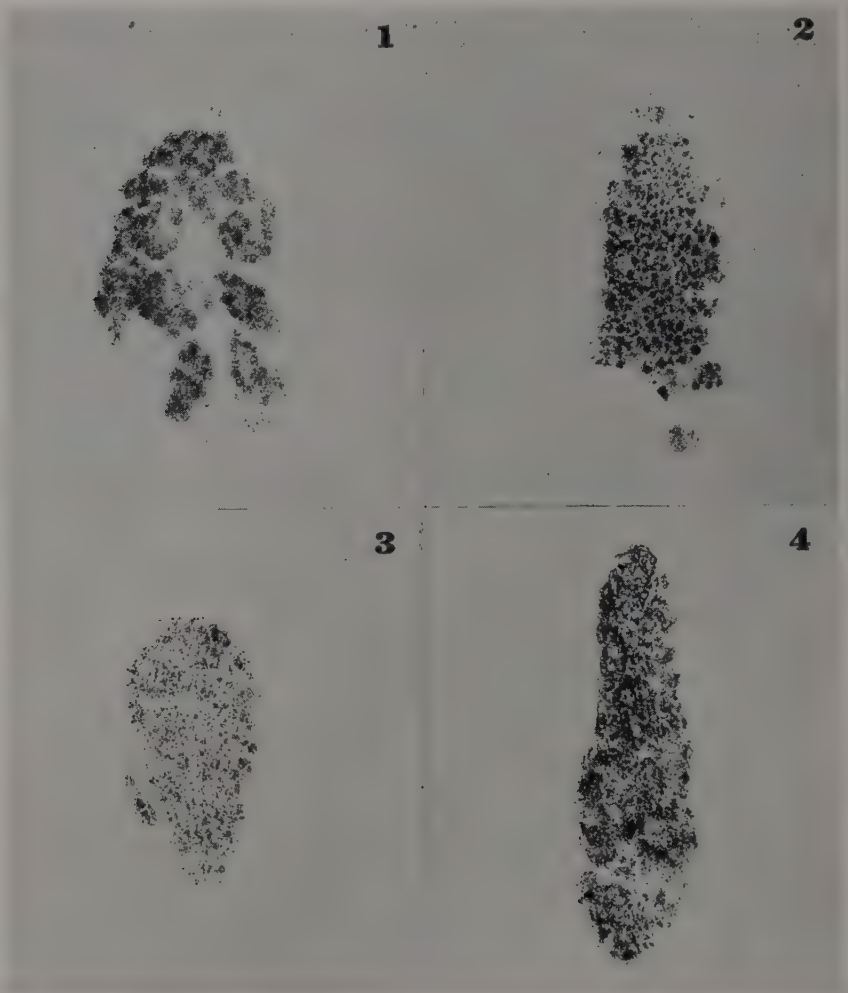
Schaunenstein and Spaeth (1859) demonstrated a strong iodine reaction in the amniotic fluid of rabbits after potassium iodide has been added to the mother's diet. Gusserow (1872) obtained similar findings in man. A few years later, Krukenberg (1885) after an extensive series of investigations confirmed this previous work and postulated that the permeability of the placenta varied with the duration of the gestation period, and that the closer to term the more readily was iodine transferred across the placental membrane. This concurs with Mossman's work (1925) and furthermore demonstrates that, near the end of gestation in the rabbit, only the fetal endothelium forms the principal barrier between fetal and maternal blood streams.

The beginning of the twentieth century brought a developing awareness of the therapeutic value of iodine. Thus, it was found that supplementary dietary iodine in pregnant sheep markedly reduced the high fetal mortality due to "fetal athyreosis." Similar results were obtained with newborn calves and colts. Following the same trend, Smith (1917) prevented fetal myxedema in pigs by adding one gram of potassium iodide daily to the mother's diet. The remarkable reduction of the incidence of cretinism in the Swiss canton of Appenzell by simply giving the pregnant mother adequate supplementary iodine climaxed this period of empiric therapeutic treatment (Curtis and Fertman, 1943).

Actual quantitative increases in the iodine content of the fetal thyroid gland following additional dietary iodine for the mother were obtained by Hudson (1931) who used finer analytical methods than had been previously available. To Hertz, Roberts, and Means (1938) should go much of the credit for pioneer work using radioactive iodine as a new tool in the investigation of thyroid physiology. The first good auto-radiographs of the human thyroid gland in both normal and abnormal states were those of Hamilton, produced in 1942. Using the same technique, Gorbman and Evans (1943), showed that the fetal rat does not significantly accumulate maternally injected radioiodine until the eighteenth or nineteenth day of its twenty-one day gestation period. This fetal stage corresponds to the time of appearance of definitive thyroid follicles and the accumulation of colloid within them.

The transfer of known chemical substances from mother to *nursing* offspring has also been the subject of extensive study. Obstetricians have long been aware of the effect upon the nursing child of the maternal intake of such drugs as alcohol, morphine, cathartics, ether, etc. (DeLee and Greenhill, 1947). In 1908 Reijst-

Scheffer showed that the feeding of potassium iodide to cows increased the iodine content of the whey; and Scharrer (1927) showed that adding the same substance to the diet of the lactating goat could result in a twenty-fold increase in the iodine content of its milk within thirty minutes. In the human infant, under normal

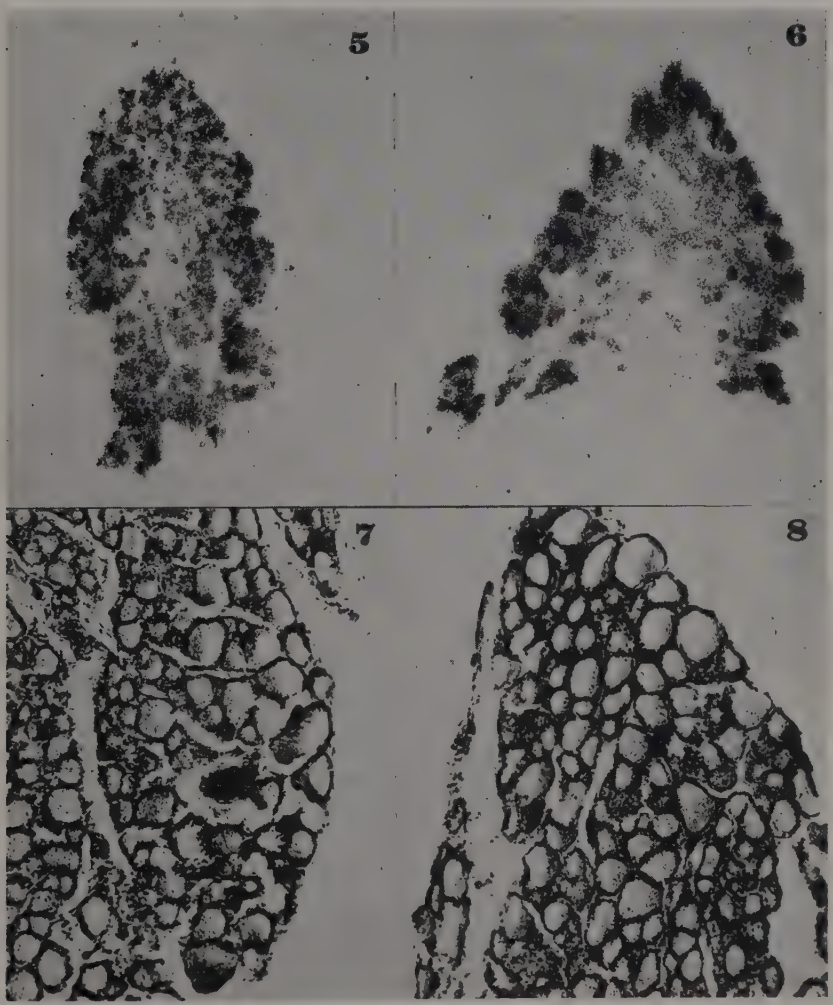


FIGURES 1–4. Auto-radiographs of thyroids of pre-nursing rats.
FIG. 4. Thyroid of still-born rat.

conditions, the intake of iodine by way of breast milk becomes relatively high beginning on the sixth day post-partum, (Elmer and Rychlik, 1934). Using relatively high doses of radioactive iodine, Rugh (1950, 1951) found that in mice the lactating mammary glands serve as excretory channels for the elimination of extra iodine intake, and that nursling mice have a high uptake of the iodine in their thyroid glands.

Much less voluminous are the investigations relating to the passage of substances across the placenta from *fetus* to mother. Snyder (1943) has found that intrafetal

injections of certain hormones, among which are pituitrin, anterior pituitary extract, and parathyroid extract fail to reach the maternal circulation or to have any effect on the maternal economy, whereas phenolsulphonephthalein was transferred readily across the placental barrier.



FIGURES 5-8. Auto-radiographs of thyroids of several nursing rats.

MATERIALS AND METHODS

Part 1. Two groups of six rats each were used. All individuals of Group I were pregnant rats near term. Group II rats were in a post-partum lactating condition. Each adult animal was kept in its own individual cage and each received a single subcutaneous injection of radioactive, carrier-free iodine of eight day half-life (I^{131}).[1] The dose of radioiodine given was calculated on the basis of one micro-

[1]Radioactive iodine was furnished through the Atomic Energy Commission, Oak Ridge, Tenn., and Dr. Wm. Myers, Dept. of Physiology, The Ohio State University, Columbus, Ohio.

curie for every ten grams of body weight. This amount is comparable to the therapeutic doses being given to human beings.

Group I (pregnant) rats received the I ^{131} from one to three days prior to the estimated day of parturition. The newborn of this group were all sacrificed before their first nursing attempt. These newborn animals are therefore designated as pre-nursing offspring.

Group II (lactating) rats received the injection of I ^{131} a variable time after delivery—from within a few hours to several days. The young were sacrificed after having nursed one or more times. These new-born animals are therefore designated as *nursing offspring.*

Stained and unstained histological sections were prepared of the thyroid glands of all the sacrificed animals of both groups. Autoradiographs of these sections were prepared after the technic of Bélanger and Leblond (1946), and their associates, (Leblond, Percival and Gross, 1948). This method consists essentially of coating the sections with a senstive photographic emulsion, followed by a suitable exposure time. The slides are then developed. The deposition of tiny black granules (silver) upon the histological sections indicates the approximate location of the disintegrating radioactive particles in the tissues. Owing to varied exposure time intervals elapsing before developing the different slides, the intensity of granular blackening must not be construed as representing a quantitative index of the uptake of radioiodine in these sections.

To demonstrate that the lactating rat breast serves as an excretory mechanism for excess iodine, as Rugh (1950) has shown for the lactating mouse, 100μc. radioiodine (I ^{131}) was added to the drinking water of a rat which had been nursing its litter. The nursling rats were removed and the mother was sacrificed the next day. The maternal mammary glands were removed and each disclosed a relatively high degree of radioactivity. Several of the glands were then thinned out by stretching and whole mount contact x-ray auto-radiographs were made (fig. 11). Due to the leaching out properties of unfixed iodides during the usual preparation of microscopic sections it was found advisable to use this gross section contact method with non-screen x-ray film.

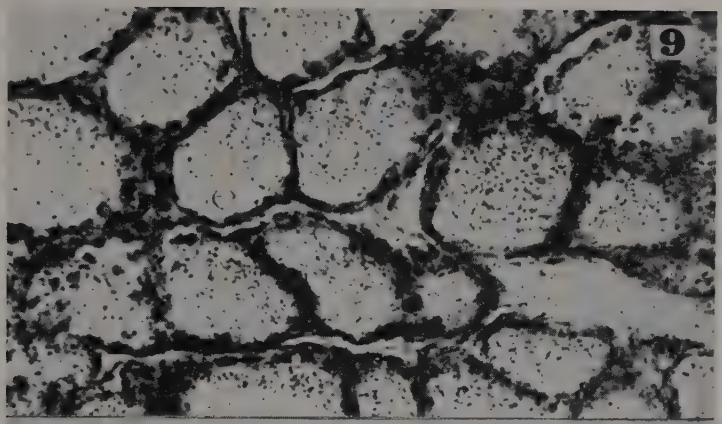
Part II. Three pregnant rabbits each near term were used. The animals were anesthetized, the abdomen opened, and a single fetus of each of the three litters received one injection of radioactive iodine (I ^{131}) in the amount of 210, 500 and 500 μc respectively. The radioiodine was mixed with a small amount of India ink just prior to injection. The abdominal wound was then closed and about twenty-four hours later the three pregnant rabbits were sacrificed. All the fetuses, including the three that were injected were found to be alive. The thyroid glands of the mothers and those of the fetuses were prepared in the usual manner for histological sections and for auto-radiography, employing the photosenstive emulsion coating method (Leblond, et al., 1948). Preliminary radioactivity assay with the Geiger counter disclosed considerable activity in all the thyroid gland material. The three fetuses which received the injected radioiodine were easily identified by the India ink discoloration and their thyroids were more radioactive than the others. All the autoradiographs revealed the typical picture of radioiodine uptake with concentration in the colloid. (figs. 12–17)

EXPLANATION OE FIGURES, 9, 10, 11.

FIGURE 9. Higher magnification of a portion of FIG. 8.

FIGURE 10. Auto-radiograph. Cross-section of trachea and surrounding structures, nursing rat. Note the absence of radioiodine uptake in parathyroid gland, A.

FIGURE 11. X-ray film auto-radiograph, gross section contact method. Lactating mammary gland of rat. Radioiodine administered in drinking water.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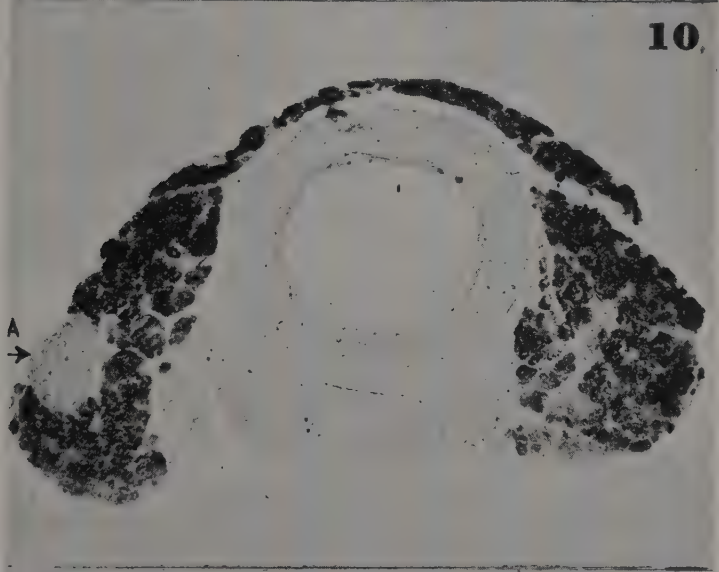
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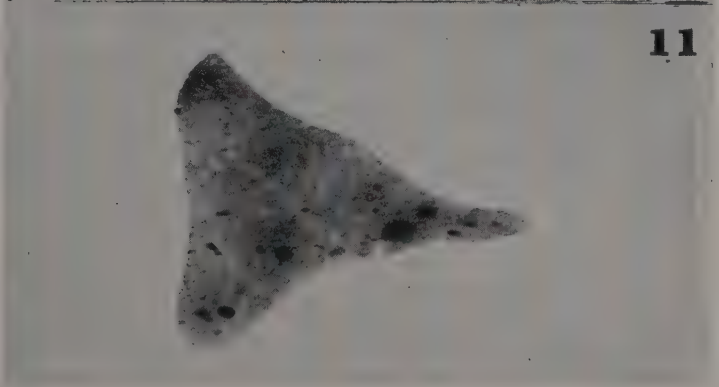
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RESULTS

Part I. All the auto-radiographs of the thyroids of both the pre-nursing and the nursing newborn rats showed considerable deposition of the black silver granules over the thyroid follicles. This reveals an appreciable uptake of the maternally adminstered radioiodine in the neonatal thyroid glands resulting from a transfer of the radioactive iodine across the placental barrier, in the one case, and across the breast parenchyma in the other.

Figures 1–4 are auto-radiographs of the thyroids of several prenursing rats. Figure 4 is of special interest since it is taken from a stillborn rat. This rat was one of two stillborn litter-mates, there having been a total of seven siblings in this litter. The autoradiograph of one of the five litter-mates born alive is shown in figure 3. Since the thyroids of both stillborns showed considerable uptake of I^{131}, the death of these animals must have occurred some time after the radioiodine was injected into the mother. It would appear then that the finding of radioactive iodine in the thyroid of a dead fetus following maternal injection of it, would indicate that fetal death had occurred sometime *after* the injection had been made. Conversely, had there been an absence of radioiodine in the thyroid of the stillborn, under these same conditions, it would have indicated that the animal had been dead *prior* to the injection of the mother. Thus, by the use of an innocuous tracer dose of radioiodine, maternally administered, one could determine whether fetal death occurred before or following some concurrent experimental procedure. Such a deduction, however, would be valid only after the fetal thyroid gland had become morphologically, functional, which in the rat is on the eighteenth or nineteenth day of fetal life, (Gorbman and Evans 1943).

Auto-radiographs of the thyroid glands of four of the nursing group of newborn rats are shown in figures 5–8. Those in figures 7 and 8 are from an older litter than are those in figures 5 and 6.

Figure 9 is a higher magnification of a portion of figure 8, and gives more of the detail of granule distribution.

Figure 10 is a cross section of the trachea and its surrounding structures in a nursing rat at the level of the thyroid isthmus. Note the lack of radioiodine uptake in the island of parathyroid tissue (A) which is almost free of black granules as contrasted with the intense distribution over the thyroid gland.

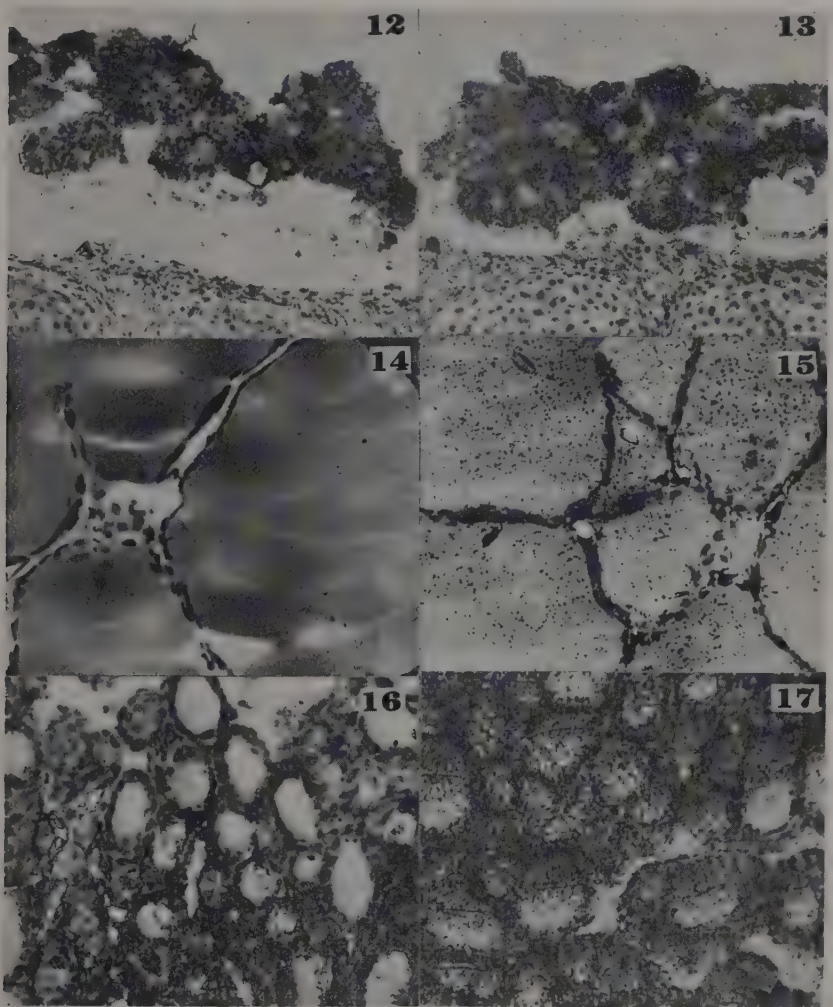
Figure 11 is a contact auto-radiograph of a lactating mammary gland of the rat that had received radioiodine in its drinking water.

Part II. All the auto-radiographs of both maternal and fetal thyroids indicated uptake of radioiodine. The accumulation of radioactive iodine in the thyroid glands of the pregnant rabbits following its injection into a fetus would indicate the transfer of that iodine across the placenta from *fetus* to *mother*. The fact that the sibling thyroids revealed considerable uptake of radioiodine indicates the transfer of radioiodine, first from the injected fetus across the placenta to the mother, and then back from the mother across the placenta to the other fetuses of the litter. There are no known vascular communications between litter mates, each fetus having its own discrete placenta. Thus the rabbit placenta in late gestation is pervious to iodine in either direction. Figures 12–17 show the histological sections and the corresponding auto-radiographs of the thyroid gland of one of the pregnant rabbits and of two of its fetuses. Histological sections are on the left, and the corresponding auto-radiograph on the right.

DISCUSSION

There is no question but that the properties of the placenta with regard to the degree and selectivity of its premeability towards different substances vary with the stage of gestation. This fact was recognized by Krukenberg in 1885, and we ought constantly to bear in mind that the placenta is not a simple membrane, but to quote Gellhorn and Regnier (1936), "it represents a complex organ which itself produces

diverse chemical transformations." Thus it is only in a limited sense that the transfer of substances across the placenta follows the general laws that obtain for diffusion across permeable membranes. Among the determining factors are those of the crystalloid or colloid nature of the molecule or molecular aggregate, the molecular weight of the substance, the relative concentrations of the material on both sides of the placenta, and their hydrogen ion concentrations.



FIGURES 12–17. Histological section on left and corresponding autoradiograph on right.
FIGS. 12 and 13. Thyroid gland of fetus injected in utero with I^{131}.
FIGS. 14 and 15. Thyroid gland of mother of above injected fetus.
FIGS. 16 and 17. Thyroid gland of litter mate of above injected fetus.

The method of auto-radiography is a relatively simple and inexpensive technique for demonstrating the presence of very small quantities of radio-active materials in certain applicable situations. For the thyroid gland it reveals localization of

uptake. Where relative quantitative data will suffice instead of absolute quantitative values, auto-radiography can offer much to save time, labor, and special equipment. The laboriously precise analytical technique of Hudson (1931) could today be easily substituted by the much simpler one of auto-radiography, for to quote Hudson: "All I proposed to show by this experiment was whether or not inorganic iodides passed through the placenta and reached the thyroid of the fetus."

It should be emphasized that the passage of iodine across the placenta or breast is not synonymous with the transfer of circulating thyroid hormone across these structures. The placenta is most certainly impermeable to albumen-bound thyroid hormone (Schlossmann 1932). Moreover, Courrier and Aron (1929) and Dorff (1934) are of the opinion that the fetus obtains practically no thyroid hormone from the mother. This situation apparently also holds for thyrotropic hormone (Aron '30), insulin (Schlossmann 1931), pituitrin, adrenalin, parathyroid hormone (Snyder 1943) and prolan B (Soule 1934). Carlson and Ginsberg (1941), however, have found that insulin is transferable across the placenta. That the nursling receives no thyroid hormone by way of the breast milk seems evident from the reports of Elmer and Rychlik (1934) and Dorff (1934). Rugh (1951) has found that the iodine present in the breast of the lactating mother mouse after injection of I^{131} is nearly all in the form of NaI. Apparently therefore, both the fetus and the infant must of necessity manufacture each its own thyroid hormone from the basic materials furnished by the mother, of which iodine is an essential component.

The increasingly widespread use of radioactive iodine as either a diagnostic or therapeutic agent impels one to add a word of extra caution if it is to be administered to a pregnant or lactating patient.

SUMMARY

1. Radioiodine I^{131} was injected into pregnant rats. The newborn were sacrificed *before* nursing. Auto-radiographs revealed a substantial collection of radioiodine in their thyroids, indicating the passage of iodine across the placenta from mother to fetus.
2. Radioiodine was injected into lactating rats. The newborn were sacrificed *after* nursing. Auto-radiographs showed a substantial uptake of radioiodine in their thyroids, indicating the passage of iodine across the breast.
3. Radioiodine was given by mouth to a lactating rat. Auto-radiographs showed a considerable accumulation of radioiodine in its mammary glands, indicating an excretory function of the lactating rat breast.
4. Radioiodine was injected into a single member of each litter of gestating rabbits. Auto-radiographs showed a substantial uptake of radioiodine in all the thyroid glands, including the maternal ones, indicating the passage of iodine across the placenta from the injected fetus to the mother, and then reversely across the placenta again from the mother to the siblings of the injected fetus.
5. Radioiodine may be of value from an investigative standpoint in determining whether fetal death occurred before or after a given experimental procedure.
6. Diagnostic or therapeutic use of radioactive iodine in the pregnant or lactating human subject merits careful consideration of its possible effect upon the young follicular cells of the developing thyroid gland of the fetus or new-born infant.

Grateful acknowledgment is given to Mrs. Ruth Sclar and Miss Virginia Schueller for their laboratory assistance.

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NEW ERYTHRONEURA (ERATONEURA) OF THE DIRA GROUP WITH NOTES

(HOMOPTERA: CICADELLIDAE)

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The Maculata Group of Erythroneura has recently been given subgeneric status (Eratoneura) Young (1952). It has been divided further into groups, the bulk of the species falling into the Dira Group characterized by an aedeagus shaft which is "typically warty or at least rugose . . . and usually with the lack of paired processes." The fifteen species here described belong to this group.

The color pattern in general follows this form: Vertex with three rounded pale spots, median longer, surrounded by narrow bands of color. Pronotum with median V or Y-shaped spot and angular spot back of each eye. Scutellum with spot at tip, basal angles sometimes outlined with color. Elytra: clavi with basal anchor spot and small spot before tip; coria with spot on costal margin midway between costal plaque and humeral angle, another at anterior end of costal plaque, large spot between claval suture and Cu; cross-veins and adjacent area on longitudinal veins often colored; minute black dot in posterior margin of costal plaque and larger one in base of inner apical cell. The coloring ranges from pale orange to deep red.

In the following descriptions only salient features of external markings are mentioned, and illustrations of inner ♂ genitalia portray these characters. Figures were drawn from slide mounts in which the parts were pressed flat in diaphane. They are all drawn to the same scale.

Specimens were collected by D. J. and J. N. Knull, unless other collectors are mentioned, and types are in collection of author and some paratypes in The Ohio State University Collection.

Erythroneura (Eratoneura) accita n. sp.

Figures 10, 11, 12

Markings orange, narrow on head and pronotum, broad and fused on elytra; black spot in base of inner apical cell unusually large and round.

Near *E.* (*E.*) *vittata* Knull and Auten (1937) in form of style, but with longer aedeagus and shorter pygofer hook. Allied also to *E.* (*E.*) *affinis* Fitch from which it differs in curved posterior point of foot of style. Length: 2.8 mm.

Taken in Hocking Co., Ohio: ♂ holotype, April 12, 1948; paratypes April 12, 1945, and May 12, 1947.

Erythroneura (Eratoneura) dura n. sp.

Figures 16, 17, 18

Pale orange markings on cream background, ill-defined, fused on base of clavus.

Near *E.* (*E.*) *usitata* Beamer and *E.* (*E.*) *ingrata* Beamer, but with a smaller foot of style and straighter pygofer hook; aedeagus straighter as in *ingrata*. Length: 2.7 mm.

Ohio: ♂ holotype, Delaware Co., Oct. 1, 1944; paratype, Greene Co., April 28, 1949.

Erythroneura (Eratoneura) inksana n. sp.

Figures 4, 5, 6

Markings pale orange, ill-defined, merging.

Near *E.* (*E.*) *penesica* Beamer, but with straighter, smaller pygofer hook, posterior point of foot of style not curved, and aedeagus broader and straight in lateral view. Length. 2.7 mm.

Collected on oak in Texas: ♂ holotype, allotype, 10 ♂ and 9 ♀ paratypes, Inks Lake State Park, Burnet Co., 1 ♂ and 1 ♀, Gillespie Co., April 5, 1953; 1 ♂, Leon Co., April 6, 1953.

Erythroneura (Eratoneura) masonae n. sp.

Figures 7, 8, 9

Color markings pale orange, diffuse, connected, ill-defined throughout.

In genital characters of ♂ related to *E.* (*E.*) *univittata* Robinson and *E.* (*E.*) *basilaris* Say, but without any trace of band in external markings. Length: 3 mm.

Nebraska: ♂ holotype, Elkhorn River, May 14, 1936, D. M. Johnson. Named for Esther Mason Johnson, my mother, who has long been interested in collecting leafhoppers.

Erythroneura (Eratoneura) nimia n. sp.

Figures 1, 2, 3

Markings pale orange, broad, joined, distinct.

Related to *E.* (*E.*) *knighti* Beamer, but with longer posterior point on foot of style, and shorter aedeagus. Length: 2.8 mm.

Ohio: ♂ holotype, Hocking Co., Sept. 27, 1950.

Erythroneura (Eratoneura) rubida n. sp.

Figures 19, 20, 21

A broad pink band behind middle of elytra and before apical cells distinguishes this species externally from *E.* (*E.*) *affinis* Fitch which it resembles in characters of inner ♂ genitalia. Other markings are somewhat diffuse and pale orange on a cream background. In some specimens the pink band is not definite, but in all the body color is light throughout, whereas in *affinis* it is more or less darkened.

Pygofer hook straighter than that in *affinis*. Length: 2.8 mm.

Taken on elm in Ohio: ♂ holotype, Fairfield Co., July 26, 1945; allotype, Hocking Co., May 22, 1938; ♂ and ♀ paratypes, Columbus, Oct. 21; Delaware Co., 2 April 8, 1945, 1 April 26, 1944, 5 April 14, 1946, 6 April 18, 1948; Hocking Co., 1, May 8, 1951.

Erythroneura (Eratoneura) socia n. sp.

Figures 13, 14, 15

Markings deeper orange on head, pronotum and apex of scutellum; narrow, distinct, small and paler on elytra.

Related in shape of foot of style to *E.* (*E.*) *solita* Beamer but with heavier, differently formed pygofer hook which is very broad at base, concave on outer portion, with ventral margin toothed toward apex. Length: 3 mm.

Ohio: Hocking Co., ♂ holotype April 12, 1945; paratype, April 17, 1947.

Erythroneura (Eratoneura) solida n. sp.

Figures 22, 23, 24

Spots small, red, well separated.

Distinguished from *E.* (*E.*) *solita* Beamer by heavy pygofer hook and lack of spine at base of aedeagus. Length: 2.6 mm.

Ohio: ♂ holotype, Hocking Co., April 12, 1945; Georgia: 2 ♂ paratypes, Decatur, May 3, 1934 and April 17, 1934, Mary Auten, Collector.

Erythroneura (Eratoneura) tantula n. sp.

Figures 34, 35, 36

Markings faint, orange, diffuse.

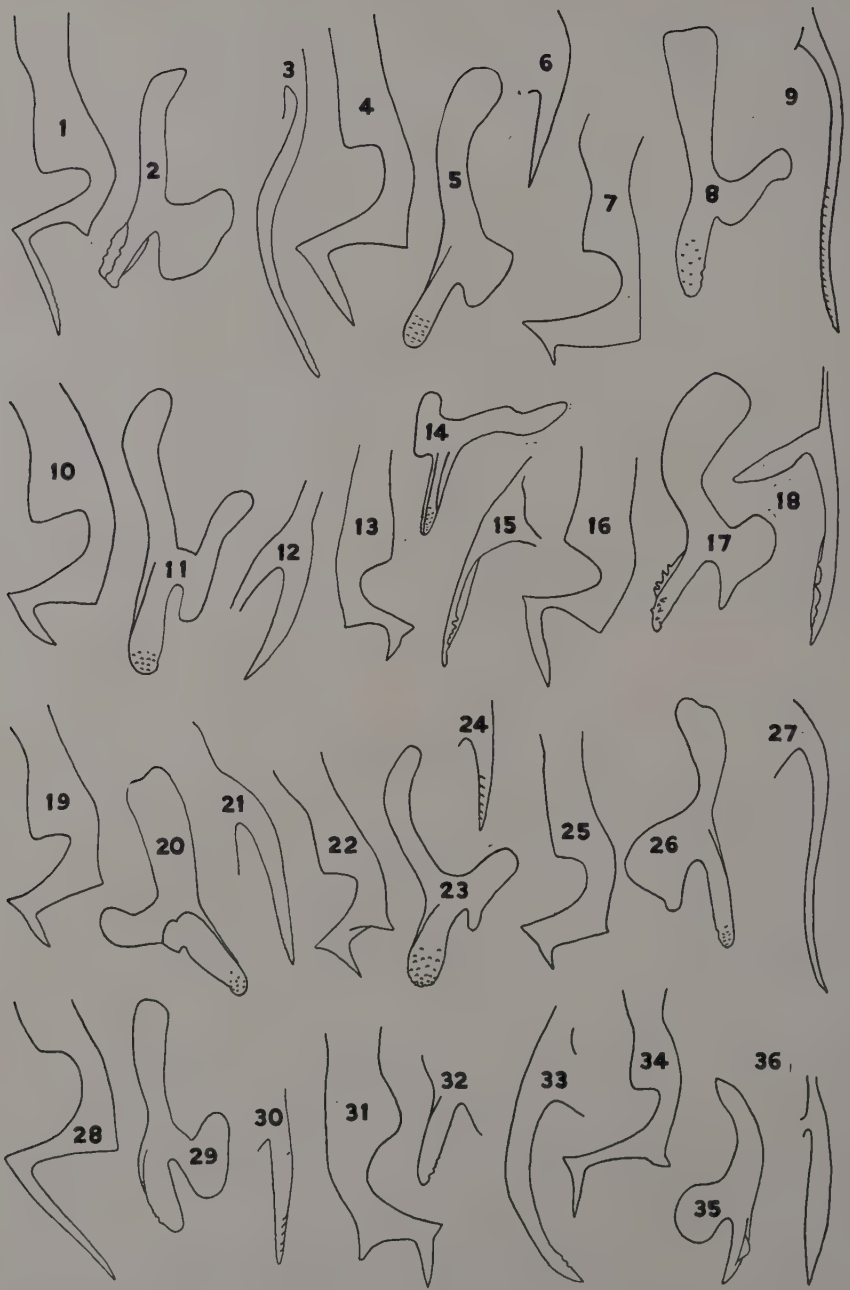
In characters of inner ♂ genitalia resembling *E.* (*E.*) *macra* Beamer, but with shorter, stubby pygofer hook. Length 2.8 mm.

Ohio: ♂ holotype, Scioto Co., June 9, 1943.

Erythroneura (Eratoneura) staminea n. sp.

Figures 40, 41, 42

Markings red on white background, small, distinct, anterior marks on clavus joined; tip of scutellum red.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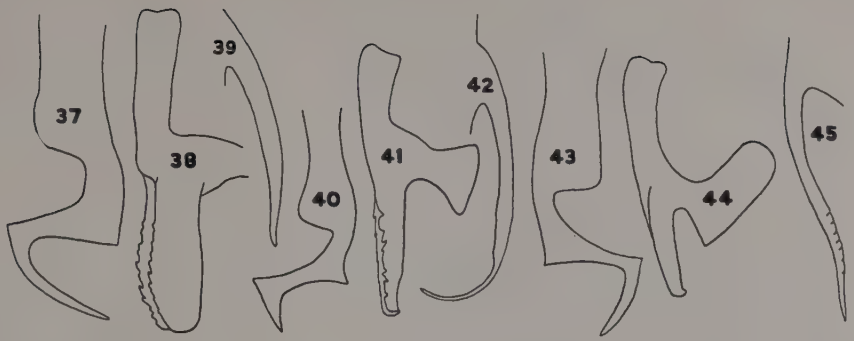

EXPLANATION OF FIGURES

In the three numbers given for each species, first applies to apical portion of style, second to lateral view of aedeagus, and third to pygofer hook.

Erythroneura (*Eratoneura*) *nimia* n. sp. 1, 2, 3.
" " *inksana* n. sp. 4, 5, 6.
" " *masonae* n. sp. 7, 8, 9.
" " *accita* n. sp. 10, 11, 12.
" " *socia* n. sp. 13, 14, 15.
" " *dura* n. sp. 16, 17, 18.
" " *rubida* n. sp. 19, 20, 21.
" " *solida* n. sp. 22, 23, 24.
" " *tenuitas* n. sp. 25, 26, 27.
" " *vacua* n. sp. 28, 29, 30.
" " *unca* n. sp. 31, 32, 33.
" " *tantula* n. sp. 34, 35, 36.
" " *tumida* n. sp. 37, 38, 39.
" " *staminea* n. sp. 40, 41, 42.
" " *valida* n. sp. 43, 44, 45.

Lash-like tip of pygofer hook distinguishes this from all others. In Beamer's key (1932) it would run to *E.* (*E.*) *interna* Beamer. This tip appears flexible, definitely curved either in or out. Length: 3 mm.

Ohio: ♂ holotype, Hocking Co., May 4, 1950; paratype, Scioto Co., June 9, 1943.

Erythroneura (Eratoneura) tenuitas n. sp.

Figures 25, 26, 27

Markings pale orange, broad, joined.

A short stubby foot and long slender straight aedeagus as well as the long slender pygofer hook characterize this species which bears some resemblance to *E.* (*E.*) *texana* Beamer (1929), however in *tenuitas* the pygofer hook is not bent and there is not a definite spine anterior to base of shaft of aedeagus. Length: 2.6 mm.

Texas: ♂ holotype and allotype, Bastrop State Park, March 26, 1950.

Erythroneura (Eratoneura) tumida n. sp.

Figures 37, 38, 39

Markings distinct, orange, joined.

Near *E.* (*E.*) *gemina* Beamer from which it differs in its very broad, large aedeagus. Posterior point of style curved strongly so tip is even with heel. Length: 3 mm.

Ohio: ♂ holotype Hocking Co., May 4, 1950, paratype October 12, 1947; 1 Pickaway Co., March 31, 1934, J. S. Caldwell, Collector.

Erythroneura (Eratoneura) unca n. sp.

Figures 31, 32, 33

Markings faint, pale orange, head darker.

Near *E.* (*E.*) *interna* Beamer but with longer posterior point on foot of style. Foot as in *E.* (*E.*) *solida* n. sp. Length: 3 mm.

Ohio: ♂ holotype, Hocking Co., May 12, 1947.

Erythroneura (Eratoneura) vacua n. sp.

Figures 28, 29, 30

Upper claval markings orange, fused, outer markings brighter red.

Near *E.* (*E.*) *abjecta* Beamer, but with longer posterior point on foot of style in proportion to base, and with broader aedeagus in lateral view. Length: 2.7 mm.

Ohio: ♂ holotype, Hocking Co., May 12, 1947.

Erythroneura (Eratoneura) valida n. sp.

Figures 43, 44, 45

Markings of medium width, orange, anterior claval spots joined.

Near *E.* (*E.*) *gemina* McAtee but with longer, more curved pygofer hook. Length: 3 mm.

Ohio: ♂ holotype, Hocking Co., May 4, 1950.

NEW OHIO RECORDS

In additon to the twelve new species from Ohio in this paper, the following have been found:

Erythroneura (*Erythridula*) *scissa* Beamer, Hocking Co., Sept. 14, 1945.

Erythroneura (*Erythridula*) *scytha* Auten and Johnson, Hocking Co., April 11, 1945.

Erythroneura (*Eratoneura*), Dira Group, *acericola* Ross and DeLong, Delaware Co., April 14, 1946; Hocking Co., April 12, 1945 and May 30, 1952; Greene Co., May 7, 1952.

E. (*E.*) Dira Group, *stoveri* Ross and DeLong, Hocking Co., May 4, 1950.

E. (*E.*), Noncuspidis Group, *eversi* Ross and DeLong, Delaware Co., April 8, 1945, April 14, 1946; Hocking Co., April 12, 1945.

E. (*E.*), Noncuspidis Group, *imbricariae* Ross and DeLong, Hocking Co., May 12, 1947.

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Introduction to Metamathematics. *Stephen C. Kleene.* D. Van Nostrand Co., Inc., New York. First edition, 1952. x+550 pp. $8.75.

The book is meant as a text book for first year graduate students in mathematics or others at that level of mathematical facility. Its aim, as stated by the author: ". . . is to provide a connected introduction to the subjects of mathematical logic and recursive functions in particular, and to the newer foundational investigations in general." This book is also being published in Holland as a volume in *Bibliotheca Mathematica.*

THE BIOLOGY OF THE PREDACIOUS MITE *TYPHLODROMUS FALLACIS* (GARMAN) (PHYTOSEIIDAE) AT 78° F.[1]

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In the spring of 1952, there appeared in the two-spotted spider mite (*Tetranychus bimaculatus* Harvey) cultures, used for miticidae research at The Ohio State University, a predacious mite of unknown identity. After a short time, the population of the phytophagus species was reduced to such an extent it was necessary to destroy the cultures and begin anew. Specimens of the predator were submitted to Dr. Edward W. Baker at the United States Department of Agriculture for identification. They were determined to be *Typhlodromus fallacis* (Garman) belonging to the Mesostigmata, Phytoseiidae.

T. fallacis was first described by Garman (1948) from collections made from apple trees in Connecticut. Garman's original description was as follows:

"*Iphidulus fallacis* n. sp. Female: 6 dorsal, 2 median and 9 lateral setae. L7 opposite M3. L9 sparsely plumose. Integument smooth, not rough or pitted. Chelicerae without teeth. Epistome of usual shape with long sharp cornicles or lateral arms and small teeth on distal margin between. Legs with one long seta on proximal segment of Tarsus IV. Sternal plate with 3 pairs of setae; anal plate reticulate, usually with 3 pairs of setae on the anterior portion (besides the para anals) and one pair of pores in addition; sometimes 4 pairs seen. One pair metapodals and 2 pairs of parapodals each side. Peritremal plates blunt and hooked at mesal ends, though not always quite as blunt as illustrated."

"Dimensions: Length .34, width .22 mm., Leg IV .32, L9 seta .03 mm. Anal plate .06 by .09 mm."

"Male: Hooks on male cheliceral appendages short, inconspicuous. Anal plate broad, with 3 pairs setae besides para anals and one pair pores."

"Dimensions: Length .25, width .16 mm."

"Type Habitat: Apple leaves. Brantford, August 11, 1947; Deep River, August 31; Hamden, August 9, 12, 19, 1947; Wallingford, August 10, 1947."

"Types in the Connecticut Agricultural Experiment Station Collection."

According to Nesbitt (1951) *Typhlodromus fallacis* has been taken from apple, raspberry, strawberry, beans, corn, and similar garden crops, dogbane, clover, and other orchard cover crops and wild trees such as the willows. It has been found in the Canadian provinces of Nova Scotia, New Brunswick, Quebec, Ontario, Manitoba, and British Columbia, and in the U. S. in Connecticut, New York, Virginia, Washington, Ohio, and California.

In these studies the predator was fed primarily *Tetranychus bimaculatus* Harvey but larvae of the European red mite *Metatetranychus ulmi* (Koch) were also found to be acceptable. Other prey as listed by Nesbitt (1951) are the four spotted spider mite, *Tetranychus canadensis* (McG.) and the Shoene spider mite, *T. schoenei* McG.

The mites were confined in a modified Huffaker cell (Peterson, 1953) which maintained a relative humidity of approximately 95 per cent. Temperature in the cell, measured by a thermacouple, averaged 78° F. It should be noted that the temperature and humidity in the cell were recorded only for a two weeks' period. Since the room and cage conditions that existed then, existed throughout rearing, it is assumed that the temperature and humidity are approximately these figures. The room temperature varied from 78° F to 81° F with an average

[1]From a paper presented at the Ohio Academy of Science which was taken from a thesis submitted to the Graduate Faculty of The Ohio State University in partial fulfillment of the requirements for the degree Master of Science.

of 80° F. Light was supplied fourteen hours per day by two 85 watt florescent tubes. All cages were usually examined four times a day.

The eggs of Typhlodromus are oval and opaque. They are laid singly, yet the female tends to place them in groups within the cell. Some adhesive material must be associated with the egg that causes it to adhere to the substrate, for an egg on the glass or leaf surface was picked up with some difficulty. The incubation period for 78 eggs averaged 46.1 hr., with the shortest time at 28 hrs. and the longest 66 hr. The average length of 15 eggs was .19 mm. and the average width .13 mm., the smallest .17 mm. by .13 mm. and the largest .21 mm. by .15 mm. (table 1)

Emergence from the egg required from 5 to 15 min. The egg covering appeared to split transversely with the larva backing out of the shell. Upon emerging, the larva rested for a short while, approximately 10 to 15 min., then moved about slowly. The extent of movement varied; some never ventured more than 3 or 4 mm. from the egg shell until they molted, whereas others explored most of their

TABLE 1

Length of life of Typhlodromus fallacis (Garman)

	No. of *T. fallacis* used in experiment	Length of Stage		
		Minimum	Maximum	Average
Adult of both sexes	57	24 da.	39 da.	32.2 da.
Mated adult female	37	24 da.	39 da.	32.8 da.
Unmated adult female	5	26 da.	39 da.	31.9 da.
Adult male	15	25 da.	37 da.	32 da.
Deutonymphal instar (female)*	48	17 hr.	37 hr.	26.3 hr.
Protonymphal instar of both sexes	67	21 hr.	47 hr.	32.6 hr.
Protonymphal instar female	48	21 hr.	40 hr.	31.5 hr.
Protonymphal instar male	19	23 hr.	47 hr.	33.8 hr.
Larval instar	76	9 hr.	26 hr.	15.4 hr.
Egg	78	28 hr.	66 hr.	46.1 hr.

*There is no deutonymphal instar for the male.

container. Although these young forms moved slowly in the normal course of events, they could move quite rapidly if disturbed. No larva was ever seen feeding but it is believed they will occasionally attack an egg. On two occasions larvae were seen near round viable appearing spider mite eggs. Upon returning to observe the predator, the eggs were seen to be crumpled, having the appearance of feeding damage, also the suspect had moved away. The larval stadium was short, averaging 15.4 hr., with a minimum of 9 hr. and a maximum of 26 hr. (table 1). In appearance the mite retained the opaque color of the egg and tended to be slightly less oval. The average size of 10 early larval instar specimens was .19 mm. by .14 mm. (All measurements exclude the gnathosoma.)

There appeared to be a short resting period before the larva molted and transformed into the second instar, the protonymphal stage. During molting, the primary cuticle appeared to split horizontally across the posterio-dorsal region of the mite and the protonymph began, by a series of twisting turning motions, to back out of the exuvium. The fourth pair of legs, which appeared at this stage, were folded in a question mark shape against the metapodosoma and slanted back towards the opisthosomal region. As molting progressed, legs IV were gradually extended. During molting, the young protonymph appeared shiny and moist as if covered by a fluid—believed to be the molting fluid. Extracation required from 10 to 25 min.; after which, there was a short period of rest, varying from 5 to 10 min. The protonymph changed after feeding from opaque grayish-white

to light straw. It was slightly more elongate and larger with an average measurement of .23 mm. by .15 mm. for 15 early protonymphal specimens. Following the post-molt rest, the protonymph became active and if the host was present, usually fed within an hour.

Males of the two-spotted mite were used as host material for all stages. Males were used because when females served as hosts, it was difficult to determine whether the mite was feeding on many eggs and an occasional female, or many females and an occasional egg; also the female spider mite cluttered up the cell with webbing, making it difficult to observe, and the female fed more extensively on the foliage than the male, thus necessitating replacement of leaves much more often.

When the male *Tetranychus* was attacked, the protonymph was often dragged a considerable distance, sometimes causing it to release its grasp and seek another prey. The predator apparently punctured the exoskeleton of *Tetranychus* and sucked out the body fluid, leaving only a shell. During the feeding act, the spider

TABLE 2

Number of Tetranychus bimaculatus Harvey consumed per day by Typhlodromus fallacis (Garman)

Typhlodromus fallacis	No. of *T. fallacis* used in experiment	No. of *T. bimaculatus* consumed per day		
		Minimum	Maximum	Average
Mated adult female	39	5.4	10.1	8.0
Unmated adult female	5	3.4	5.2	4.0
Adult male	15	2.7	5.7	3.9
Deutonymphal instar	48	2.0	11.0	6.6
Protonymphal instar	67	2.0	9.0	4.8
Larval instar	76	none*	none*	none*
Mated female (larva through adult)	39	5.1	9.8	7.5
Unmated female (larva through adult)	5	3.3	6.0	4.1
Male (larva through adult)	15	2.7	5.4	3.9

*Believe to feed occasionally.

mite was manipulated and held by the first pair of legs. Once the prey had become quiet, the first pair of legs of *T. fallacis* were raised above its body and held at an acute angle from the midline. The feeding position was often changed merely by moving the food. Mites that had been fed upon had a characteristic shrivelled appearance. From the time of attack until the male two-spotted mite was completely subdued approximated two minutes. Feeding then took from 5 to 40 min. In the shorter feeding periods, the prey was not completely consumed. Often the predator would move a short distance away from the host (about 1 mm.) appear to clean itself, and then return and resume feeding. Feeding usually did not continue from the original spot of attack but three or four areas were tried. The minimum food requirement for completion of the protonymphal instar was two males, the average was 4.8 and the maximum was 9 (table 2). The protonymphal stadium varied from 21 to 47 hr. with an average time of 32.6 hr. (table 1).

In these studies, only the female passed through the deutonymphal stage; males were adults at the end of the second instar. The deutonymph was larger and slightly more rounded than the protonymph; coloration was little changed. The average size of 15 early deutonymphs was .18 mm. by .30 mm. Prior to the protonymphal molt, there was a resting period of approximately one hour. Thus, molting was similar to that of the first instar, except four pairs of legs were present prior to ecdysis. The time consumed in shedding was from 10 to 30 min. Again there was a resting period upon completion of the molt which was about the

same amount of time as the protonymph. Upon completing the resting period, the mite became active, and within an hour started to feed. The prey was attacked from any angle. Approximately 45 to 60 sec. after the prey was seized, it had been subdued. Most of the mites attacked by the deutonymph were completely consumed, a process requiring from 8 to 15 min. The number of phytophagus mites eaten during the stadium was from 2 to 11 with an average of 6.6 (table 2). The stadium lasted on an average of 26.3 hr. with the shortest at 17 hr. and the longest at 37 hr. (table 1). Upon completion of the deutonymphal instar, the mite became an adult female. It was concluded to be an adult on two bases: it did not undergo another ecdysis after the deutonymphal molt; and mating occurred only after the deutonymphal stage.

Mating occurred within an hour after the adult stage was reached. Prior to copulation, no pre-mating activity on the part of either sex was observed. The male approached the female posteriorly and crawled upon her dorsum and faced anteriorly. He then turned and moved posteriorly over the opisthosoma, between legs IV, and under the female. They were then resting venter to venter. The male, with Leg I, clasped the female between legs I and II, with legs II between III and IV, and legs III just posterior to legs IV. Legs IV of the male then grasped the posterio-dorsal region of the opisthosoma. While obtaining the above position, the male underwent a side to side turning movement. Once in the copulatory embrace, the female remained more or less stationary. Mating was a prolonged process, usually continuing over 2 or 3 hrs. During copulation, the male exhibited periodic, jerky movements. There did not appear to be a specific number of times a particular sex would mate; one male copulated with 7 different females, all of which laid eggs, and one female copulated 5 times. One female that mated refused the male 24 hours later. Copulation evidently was not always successful. In one instance a female mated 3 times yet did not oviposit.

Prior to copulation the female was ordinarily a pale straw but after copulation became a straw color. Mating appeared to be necessary for egg production. In one test, 5 females reared from egg to adult without mating (one was reared in the presence of all host stages to examine the possibility of a nutritional influence upon female fertility) produced no eggs. One female reared through most of its adult existence without mating produced no eggs, yet upon copulation, eggs were laid.

The pre-oviposition period was approximately 24 hr. Prior to ovipositing, the egg could be readily observed in the gravid female. About an hour before egg laying, the female became quiet and remained near the spot upon which she was to lay her egg. The actual egg deposition was never observed, but a female was seen about to oviposit and upon re-examining her three minutes later, the oviposition act had been completed. After depositing her egg, the female usually moved actively about the cage. The average number of eggs per female per day was 1.5, the maximum 2.3, and the minimum .5. Most females stopped ovipositing 7 to 10 days before death. The ovipositing period lasted approximately 22 days with an average number of 2.2 eggs laid per day.

In feeding, both males and females attacked all stages. In attacking host material in the cells, the male *Tetranychus* was seized from any area; one hungry female was seen to grasp a male by the pedipalp and begin feeding. Adult male typhlodromids fed on the average of 3.9 male mites per day, and also 3.9 per day per predator life time (table 2); subdual and feeding required approximately the same amount of time as for the deutonymph. The mated adult female, which fed more extensively than the unmated female, consumed more than the male. Her per day average was 8.0 and lifetime per day average 7.5. The female was the larger of the species and was capable of subduing a male spider mite in 30 sec. and consuming him in 3 min. It was noted that both adults and immatures did not always consume all the prey attacked, but these were all left in such a condition that no recovery was ever noted.

There was little difference in length of life between the two sexes. Adult males lived from 25 to 37 days with an average of 32 days whereas adult mated females lived from 24 to 39 days with an average of 32.8 days. Both sexes, aside from ovipositing, had similar habits. The female, when old and no longer ovipositing, tended to return to the light straw color of the unmated female. The appearance of the male changed little throughout its adult existence.

In all studies made, cannibalism was observed only when the predators were exposed to prolonged starvation; this held true even when as high as 8 mites of mixed stages were placed in a 2 cm. cell. No plant feeding was noted in any of the stages. The protonymphal, deutonymphal, and adult stages have been starved to death with ample green leaf surface available and during this period phytophagy was not observed. Although the larva was not seen feeding on plant material, it was difficult to conclude whether it would or would not, as it readily completed the instar in the absence of host material.

The overwintering stage is not known. In early October of 1952, however, about 12 females were collected from apple trees in the Ohio State University Orchards. From the literature and from correspondence, it is believed that the overwintering stage is the adult female.

SUMMARY

The biology of the predacious mite, *Typhlodromus fallacis* (Garman), of the Phytoseiidae, was studied by the use of a modified Huffaker cell with *Tetranychus bimaculatus* Harvey as host material.

The female of the predator which underwent three instars, had an adult life of 32.8 days. The number of eggs laid averaged 1.5 per day per imaginal period. The ovipositing period lasted approximately 22 days with an average number of 2.2 eggs laid per day. Non-ovipositing females consumed 4 male spider mites per day while the ovipositing females consumed 8 male spider mites per day.

Mating occurred a number of times and appeared to be necessary for oviposition.

The male *T. fallacis* underwent two instars with an imaginal period of 32 days. Males consumed 3.9 male spider mites per day.

The incubation period of the egg was 46.1 hr.

The larval stage was 15.4 hr. and it did not commonly feed during this period.

The protonymphal instar lasted 32.6 hr. while the deutonymph lasted 26.3 hr. The former consumed an average of 4.8 spider mites while the latter averaged 6.6 for the stadium.

No phytophagy was noted on the part of the predator and cannibalism occurred only under starvation conditions.

ACKNOWLEDGMENTS

I wish to express my thanks to Dr. Alvah Peterson for help and suggestions during this study.

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NEW RHYNCHOPHORA II
(COLEOPTERA, CURCULIONIDAE)

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The following Curculionidae were collected by, given to, or sent for identification to the author over a period of several years. This is the third of a series of papers to be published on new Rhynchophora.

LIST OF ABBREVIATIONS

DJ & JNK.....Dorothy J. and J. N. Knull.
ELS.....E. L. Sleeper.
(ELS).....E. L. Sleeper Collection.
JNK.....J. N. Knull.
(OSU).....Entomological Collection, The Ohio State University.

Smicraulax arizonicus n. sp.
Figures 2 and 5

Male:—Oblong, dull black, elytra with a reddish-brown fascia behind humeri; funicle, tarsi, and tips of femora light reddish-brown; sparsely clothed with narrow white scales, fascia of elytra densely clothed with long, yellow, narrow scales.

Rostrum, black, longer than pronotum; with narrow median carina in basal half; laterally densely punctate, coarser in basal half. Antennae inserted at middle. Funicle seven-segmented; darker toward club. Club black, elongate-ovate, densely clothed with fine, white pubescence.

Head with shallow, coarse punctures; a round deep fovea above eyes at vertex. Eyes prominent, coarsely granulate.

Prothorax strongly convex, black, apical margin reddish, sides strongly arcuate; coarsely, closely punctate; sparsely clothed with intermixed yellowish-white and brown narrow scales. Scutellum rounded, prominent, densely clothed with short, narrow, white scales.

Elytra elongate, sides subparallel in basal two-thirds, slightly wider than pronotum; humeri prominent, black; striae impressed, strial punctures wider than striae, rounded, deep; intervals strongly alutaceous, sparsely clothed with intermixed, fine, dirty white hairs and long narrow white scales, interval three with a large, prominent tubercle, elevated throughout basal fourth of interval; fascia disappearing at lateral margins of elytra, not extended backwards along margin.

Ventral side convex, abdominal segments strongly alutaceous, sparsely clothed with fine, white pubescence. Legs sparsely clothed with fine, white pubescence. Anterior femora elongate, thickened, rugosely punctate, with a broad triangular tooth mesially. Anterior tibiae flattened, broad, rugosely punctate, sinuate mesially, broadest at middle, tip unguiculate. First segment of tarsi as long as following two. Claws toothed.

Length 2.5 mm., width 1.2 mm.

Type Locality.—Unique holotype, male, Chiricahua Mts., Ariz. IX–20–48, C. W. Jones in (ELS).

Closely related to *S. tuberculatus* Pierce, but the latter has elytral fascia extending backwards along margin to apex of elytra; rostum, humeral area, femora (darkened at middle) and tibiae reddish-brown; the femora with a small narrow tooth.

Trichobaris major Barber

Trichobaris major Barber, 1935, p. 14.

Described from Durango City, Mexico, recorded from other localities in Northern Mexico, has been collected in large numbers near Fort Davis, in Jeff Davis County, Texas, in March.

T. major may be separated from our other species of *Trichobaris* by the following characters: last ventral segment medially carinate at apex in female; female rostrum shorter than pronotum, much longer and less curved than in male; it is a much larger species.

Trichobaris bridwelli Barber

Trichobaris bridwelli Barber, 1935, p. 23.

Described by Barber in 1935 from South Carolina, Georgia, Alabama, Louisiana, Missouri, Oklahoma and Texas, and should be added to our list of North American Coleoptera.

Centrinaspis patagoniensis n. sp.

Figures 1 and 3

Female.—Elliptical, black, shining moderately clothed with narrow, white scales; elytra with a subtriangular patch of broader white scales each side of scutellum; a black patch of scales behind scutellum.

Rostrum one-third longer than pronotum, moderately stout, strongly arcuate, abruptly bent back near base; smooth at middle, coarsely, densely punctate laterally. Antennae inserted at middle; second segment of funicle half again as long as third, 3–6 equal. Club ovate, finely, densely pubescent.

Head finely punctate; transversely grooved between eyes at base of rostum.

Prothorax strongly convex, sides arcuate, converging to constriction at apex; densely, coarsely punctate on pronotum, punctures confluent laterally, a smooth median line from base to apex; clothed with transversely arranged narrow, white scales, denser before scutellum. Scutellum rhomboidal, longitudinally impressed, denuded.

Elytra triangular, slightly wider than pronotum, sides converging to apex, widest behind humeri; not impressed along suture; striae deeply impressed, punctures very small, shallow; intervals with a double line of deep punctures; each interval with a double line of narrow white scales; black patch of scales behind scutellum occupy intervals 1 and 2 of middle disc.

Ventral side densely clothed with broad scales except at middle of abdominal segments 3, 4, and 5 which are denuded. Prosternum deeply sulcate, densely, coarsely punctured. Abdominal segments finely, densely punctate except at middle of segments 3 and 4; fifth abdominal segment with coarser punctation and a shallow depression at middle. Legs clothed with narrow, white scales. Claws large, divergent, simple.

Length 4.8 mm., width 2.5 mm.

Male.—Rostrum bisulcate anterior to point of insertion of antennae, lateral punctation coarser. Prosternum deeply sulcate, armed with two short spines; first and second abdominal segments broadly depressed at middle, depression densely clothed with erect, white pubscence; fifth abdominal segment without depression at middle.

Type Locality.—Holotype, female, Patagonia Mts., Ariz. VIII–7–50, DJ & JNK. Allotype same data. Both in (OSU). Nineteen paratypes: 12 same data as types in (OSU); 6 same data in (ELS); 1 Nogales, Ariz. IX–2–38, DJ & JNK. in (OSU).

Found on the flowers of *Acacia sp.* in the Patagonia Mts.

Closely related to *C. basinotata* (Champion) from the mountains of Guerro, Mexico. *C. basinotata* differs in that it lacks the black patch of scales, has on each interval "a narrow line of fuscous scales, intermixed with larger ones," and the first abdominal segment only is depressed in the male.

Centrinogyna hispidula Casey

Centrinogyna hispidula Casey, 1920, p. 463.

Examples of this species previously known only by the type series from the Sierra Madre Mts., Chihuahua, Mexico, have been collected in the Huachuca Mts., Ariz., VIII–12–50 to VIII–19–50, DJ & JNK.

Oblong, convex, dark reddish-brown, elytra lighter, rostrum black; vestiture of long, recumbent, whitish hairs, distinct on prothorax, denser on sides; very dense on third intervals of elytra, sparse on remaining intervals.

Females are much more abundant than males. In the males pygidium is vertical, exposed and strongly convex; first abdominal segment with a narrow, shallow median depression.

In general outline resembling *C. strigata* (LeConte), but otherwise unlike any other member of the genus. The peculiar vestiture sets it apart from any other Barinid.

D. J. and J. N. Knull collected many examples on the tips of blades of grass in Miller Canyon, Huachuca Mts., Arizona at approximately 5500 ft.

Eulechriops pictus Schaeffer

Eulechriops pictus Schaeffer, 1908, p. 219.

A single example of this beautiful and rare weevil was collected in the Patagonia Mts., Arizona VII–15–52 by DJ & JNK. The type and all other known examples were collected in the Huachuca Mts., Ariz. The afore mentioned example was kindly given the author by Professor Knull.

Conotrachelus cameronensis n. sp.

Female.—Oval, robust, black, antennae tibiae and tarsi reddish-brown; with yellowish-white, narrow, recumbent scales and erect intermixed white and brown hair-like scales; prothorax without recumbent scales.

Rostrum slightly longer than prontum, above a smooth, narrow carina; laterally deeply sulcate, forming lateral carinae, coarsely, rugosely punctate basally; sparsely clothed with short, white bristles. Antennae inserted in apical fourth; scape not attaining eyes; funicle seven-segmented, first two segments subequal, second as long as three and four combined, segments 3–6 round subequal. Club ovate, densely, finely pubescent.

Head coarsely, closely punctate; between eyes a deep transverse groove. Eyes flat, coarsely granulate.

Prothorax feebly convex, sides subparallel in basal three-fourths, thence strongly narrowed; coarsely, deeply, closely punctate; sparsely clothed with intermixed, erect, yellowish-white and and brown hair-like scales. Scutellum rounded posteriorly, elevated, flattened dorsally, without vestiture.

Elytra oval, one half wider than pronotum, sides parallel in basal half, thence converging to apex; humeri very prominent; striae not impressed, strial punctures small, round, deep about their own diameter apart, each bearing a very narrow, white scale; intervals flat on disc, intervals 3, 5, and 7 strongly convex behind declevity; vestiture of narrow, recumbent yellowish-white and white recumbent scales; each interval with a single row of intermixed white and brown erect hair-like scales.

Ventral side very sparsely clothed with narrow, white scales; abdominal segments closely, coarsely punctate; fifth abdominal segment with a deep crescent-shaped depression at middle. Legs moderately clothed with vestiture similar to ventral segments. Femora robust, closely, coarsely punctate, with a broad tooth mesially. Tibiae feebly flattened, feebly sulcate longitudinally, straight mesially. Claws with long teeth.

Length 4.4 mm., width 2.3 mm.

Male.—Fifth abdominal segment flat, not impressed at middle.

Type Locality.—Holotype, female, Cameron Co., Texas V–20 in (ELS). Allotype same data in (ELS). Eleven paratypes: 1 Brownsville, Texas V-31–39 DJ & JNK., 1 Brownsville, Tex. V–25–34, JNK., 2 Brownsville, Tex. VI–8–34, JNK., 3 Brownsville, Tex. V–22–35, JNK., 4 Brownsville, Tex. V–15–35, JNK. in (OSU).

Closely related to *C. texanus* Schaeffer for which it has been mistaken. *C. cameronensis* differs from the types of *texanus* as follows: larger, more elongate; striae of elytra not impressed (in *texanus* deeply impressed), punctures of pronotum finer; erect scales longer, recumbent scales much narrower.

Ryssematus texanus n. sp.

Female.—Oval-elongate, black, feebly, shining, elytra reddish-black, mottled with black spots; sparsely clothed with yellow, recumbent hair-like scales.

Rostrum longer than pronotum, finely carinate at middle, bicarinate laterally in base; densely finely punctate throughout. Antennae reddish-brown, funicle seven-segmented, first segment longer than second, second longer than third and fourth combined. Club ovate, finely pubescent.

Head very convex, coarsely, deeply, densely punctate; sparsely clothed with yellow hair-like scales. Eyes coarsely granulate, very narrowly separated above.

Prothorax wider than long, feebly convex, sides arcuate to strong apical constriction; pronotum carinate in apical two-thirds, numerous oblique strigae radiating therefrom; sparsely clothed with yellow hair-like scales. Scutellum densely clothed with white pubescence.

Elytra slightly wider than pronotum; striae not impressed, punctures large, deep, wider than striae, encroaching on intervals; odd intervals acutely carinate; sparsely clothed with yellow hair-like scales.

Ventral side sparsely clothed with recumbent yellow hair-like scales, densely punctate. Legs clothed as ventral side. Femora robust, very thick, rugosely punctate, with a broad tooth mesially. Tibiae broad, flat, longitudinally sulcate, a row of white scales in each sulcus. Claws strongly toothed.

Length 5.1 mm., width 3.1 mm.

Type Locality.—Holotype, female, McLemon Co., Texas VII–25 in (ELS). Allotype Cameron Co., Texas V–30 in (ELS). Fourteen paratypes: 1 Cameron Co., Texas V–30, 1 Brownsville,

1. *Centrinaspis patagoniensis* n. sp., ♀.
2. *Smicraulax arizonicus* n. sp., ♂ (Holotype).
3. Lateral view of rostrum, head, and prothorax of *Centrinaspis patagoniensis* n. sp., ♂.
4. Anterior femur of *Smicraulax tuberculatus* Pierce, ♀.
5. Anterior femur of *Smicraulax arizonicus* n. sp., ♂ (Holotype).
6. *Paracamptus floridanus* n. sp. (Holotype).

(Line = 1 mm.)

Tex. V–25–39, DJ & JNK., in (ELS); 1 Brownsville, Tex. V–25–39–, DJ & JNK., 1 Brownsville, Tex. V–31–39, DJ & JNK., 2 Brownsville, Tex. V–8–35, JNK., 4 Brownsville, Tex. V–15–35, JNK., 3 Brownsville, Tex. V–22–35, JNK. in (OSU); 1 Brownsville, Tex. V–15–35, JNK. in (ELS).

Nearest *R. sculpturatus* Champion from Mexico, but broader with coarser vestiture and longer rostrum.

Paracamptus floridanus n. sp.

Figure 6

Elongate-narrow, reddish-brown, densely covered with a gray crust with sparsely placed erect, clavate, gray scales.

Rostrum shorter than pronotum, robust, flattened, denuded in apical three-fourths; coarsely, closely (almost confluently) punctate throughout. Antennae light reddish-brown; funicle seven-segmented, first two segments subequal, elongate.

Head strongly rounded, densely covered with gray crust, numerous erect, clavate scales, punctation completely obscured. Eyes lateral, widely separated, coarsely granulated.

Prothorax feebly convex, longer than wide, sides feebly rounded in basal three-fourths; pronotum prolonged over head, apical margin strongly arcuate, a feeble transverse depression behind apical fourth. Scutellum round, flattened not elevated.

Elytra elongate-cylindrical, as wide as pronotum; sides subparallel in basal two-thirds; striae feebly impressed almost obscured by gray crust; intervals flat, none elevated, all intervals with sparse erect, clavate scales; vestiture uniform gray with a brownish-gray W-shaped fascia at middle of disc.

Ventral side densely clothed with gray crust and gray, semierect, clavate scales; pectoral groove deep, ending between middle coxae; punctation of abdominal segments obscured by crust; second and fifth abdominal segments longer at middle than third and fourth combined. Legs clothed as ventral side. Femora very robust, unarmed. Tibiae robust, short, nearly three-fourths as long as femora, a prominent hooked spine at apex. Third tarsal segment broadly bilobed. Claws divergent; simple.

Length 1.5 mm., width 0.6 mm.

Type Locality.—Unique holotype Big Pine Key III–18 in (ELS).

Easily separated from *P. subtropicus* Casey by size, form of the fascia on the elytra, and the absence of elevated intervals on the elytra. One of the smallest of our North American cryptorhynchids.

Gerstacekeria knullorum n. sp.

Female.—Elongate-oval, black, antennae dark reddish-brown; densely clothed with black, white and rust colored scales.

Rostrum as long as pronotum; densely clothed with rust and white scales obscuring punctures in basal two-thirds; deeply, densely, coarsely punctate with a smooth median line. Antennae inserted at middle; second segment as long as third and fourth; all segments densely pubescent. Club oval, finely, densely pubescent.

Head coarsely, closely punctate; punctures nearly obscured by closely placed rust and white scales; with a shallow, elongate fovea between eyes.

Prothorax slightly wider than long, sides strongly rounded, apex slightly constricted; densely, coarsely, deeply punctate; with scattered, dirty white and rust scales. Scutellum not visible.

Elytra-oval, scarcely one-third wider than pronotum; humeri obsolete; strial punctures coarse, deep, closely placed; intervals raised, convex; with two rust and white fascia, anterior one immediately behind humeri, interrupted at middle, posterior fascia at declevity complete; suture in apical third densely covered with rust scales.

Ventral side moderately clothed with white, rust, and black scales. Abdominal segments coarsely, densely deeply puctate. Legs densely clothed with white and rust scales. Femora unarmed. Third tarsal segment not wider than second. Tarsal claws large divergent, simple.

Length 6.2 mm., width 3.2 mm.

Type Locality.—Holotype, female, Huachuca Mts., Ariz. VIII–12–50, DJ & JNK. in (OSU). Allotype Huachuca Mts., Ariz. VIII–19–50, DJ & JNK. in (OSU). Ninety-six partypes: 46

Huachuca Mts., Ariz. VIII–12–50, DJ & JNK. in (OSU); 10 same data in (ELS); 35 Huachuca Mts., Ariz. VIII–19–50, DJ & JNK. in (OSU); 5 same data in (ELS).

DJ & JNK. found this insect in large numbers on the light colored walls of a cabin in Miller Canyon.

This species superficially resembes *G. basalis* (LeConte) but keys out to *G. alternatus* Pierce in his key to the species (1912, p. 160, 162). However, *knullorum* is much smaller and in no way resembles the latter. *G. knullorum* can be separated easily from *basalis* as the latter has the third tarsal segment much larger than second. *G. knullorum* is a link between the subgenera *Gerstaeckeria* Champion and *Philopunta* Pierce, having characters which link it to both groups.

It is with great pleasure I name this species after Professor and Mrs. J. N. Knull whose annual collecting trips to the Southwestern United States have added much to our knowledge of the insect fauna of that region.

Pseudacamptus texanus n. sp.

Elongate, black, dull, densely clothed with recumbent brown scales and broad, erect, short, clavate scales throughout; all intervals strongly elevated.

Rostrum short, stout, three-fourths as long as pronotum, subcylindrical, feebly curved, longitudinally foveate throughout; encrusted with recumbent brown scales and erect clavate scales. Antennae dark reddish-brown, inserted in apical third; funicle six-segmented, first segment swollen. Club small, ovate, with shining, subglabrous basal segment; remaining segments short, sensely pubescent.

Head feebly convex, coarsely, densely punctate; a deep puncture between eyes. Eyes lateral, small, flat, completely concealed in repose.

Prothorax as broad as long, sides subparallel in basal half, strongly constricted behind apex; coarsely, densely punctate; moderately clothed with erect, robust, short, clavate scales, without recumbent scales. Scutellum small, rounded, convex.

Elytra elongate, wider than pronotum, sides parallel in basal two-thirds; striae unimpressed, punctures deep, coarse, quadrate; intervals densely clothed with recumbent scales intermixed with erect scales; intervals 2 and 4 becoming feeble beyond declevity, remainder strongly elevated dorsally, laterally intervals 10 elevated in basal fourth, 11 flat, remainder elevated.

Ventral side sparsely clothed with recumbent scales; prosternum deeply sulcate; first and second abdominal segments coarsely, closely punctate, remainder coarsely, distantly punctate; first abdominal suture nearly obsolete; fifth abdominal segment longer than third and fourth. Legs densely clothed with intermixed recumbent and erect clavate scales; densely, coarsely, closely punctate. Femora robust, thickened, unarmed. Tibiae sinuate mesially, broadest at middle; strongly unguiculate at outer apical angle and toothed at inner angle. Claws divergent, simple.

Length 3.3 mm., width 1.5 mm.

Type Locality.—Unique holotype, Colorado Co. (southeastern corner), Texas, VI–10, in (ELS).

This is probably frequently mistaken from *Acamptus rigidus* LeConte from which it may be separated by the number of segments in the funicle. From *P. plurisetosus* Champion *texanus* differs by the more strongly elevated elytral intervals, denser punctation of ventral abdominal segments and more elongate-narrow form.

Anchonus blatchleyi n. sp.

Male.—Oblong-ovate, black, dull, irregularly, coarsely punctate above, each puncture bearing a short, thick yellowish scale, all intervals, except sutural, strongly tuberculate

Rostrum slightly longer than pronotum, strongly constricted at base, strongly arched, deeply, coarsely punctured, finer at apex, each puncture bearing a short, thick scale. Antennae inserted in apical third; funicle 8–segmented, first segment short, second elongate. Club oval, densely, finely clothed with short hairs, with sparser, intermixed long hairs.

Head globose, smooth, finely alutaceous. Eyes inferior, flat, granulate.

Prothorax slightly wider than long, sides strongly rounded, strongly constricted at apex; closely, coarsely, deeply, punctate; laterally areas between punctures strongly raised into tubercles. Scutellum absent.

Elytra elongate-oval, one-half wider than pronotum, sides arcuate; humeri nearly obsolete; striae unimpressed, punctures large, round, deep; sutural interval flat, remainder feebly carinate,

three and five more strongly elevated basally, more strongly carinate throughout; each tubercle with very short bristle-like scale.

Ventral side coarsely, closely punctate, each puncture with a short, clavate scale; first abdominal suture nearly obsolete, fifth segment longer than third and fourth. Legs rugosely punctate. Femora sparsely clothed with short, robust scales. Tibiae with very sparse, long white pubescence.

Length 4.3 mm., width 2.3 mm.

Type Locality.—Holotype, male Dade Co. (near Matheson Hammock), Florida, IV–20 in (ELS). Allotype same data in (ELS). Two paratypes, Dade Co., Fla., XII–1 in (ELS).

Not close to any other species in the genus in Florida, but nearer *A. floridanus* Sz. than *A. duryi* Blatchley, separated by the strong elevation of third and fifth elytral intervals basally, stronger elevation of all intervals, and more densely tuberculate.

Named for W. S. Blatchley, a great Coleopterist and student of Florida Curculionidae.

Pentarthrinus ilicis n. sp.

Female.—Elongate, subcylindrical, black, shining, antennae, legs and tarsi dark reddish brown, club yellowish-brown.

Rostrum more than half as long as pronotum; coarsely, closely punctate in basal half, fine in apical half. Antennal scape attaining middle of eyes; funicle five-segmented, first segment moderately large, not concealing second. Club elongate, basal half subglabrous, remainder sparsely pubescent.

Head convex, alutaceous, sparsely, coarsely punctate; not foveate between eyes. Eyes small, convex, finely granulate.

Prothorax longer than wide, oval, sides evenly arcuate; densely, coarsely, deeply punctate, interspaces alutaceous. Scutellum round, flat, a deep puncture laterally each side of middle.

Elytra elongate sides subparallel in basal three-fourths; striae deeply impressed; strial punctures deep, round, their own diameter apart; intervals narrow, as wide as striae, flat, each with a single row of fine punctures twice their diameter apart.

Ventral side feebly convex, coarsely, deeply punctate. Legs sparsely punctate. Anterior tibiae strongly unguiculate at outer apical angle, feebly mucronate at inner angle.

Length 2.2 mm., width 0.8 mm.

Type Locality.—Holotype, female Pokomoke City, Md., XII–10–50, in (ELS). Allotype, same data, in (ELS). Two paratypes, same data, in (ELS).

Dug from the wood of a prone, dead holly tree (*Ilex opaca* Ait).

Close to *P. parvicollis* Casey which, however, has the interspaces of the prothorax alutaceous, prothorax shorter, as broad as long; punctures of striae smaller, of intervals very minute, and in general more narrowly elongate.

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EFFECT OF GONADOTROPIC HORMONES ON HYPOPHYSECTOMIZED (ANTERIOR LOBE) MALE *RANA PIPIENS*

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The reaction of normal male and female amphibia to gonadotropic hormones and to other reagents has received considerable attention within recent years. This study has undoubtedly been stimulated by the importance of the role these animals assumed in pregnancy diagnosis. The successful use of *Rana pipiens* in particular, as a test animal for pregnancy diagnosis has been demonstrated by Wiltberger and Miller (1948). The procedure has as its basis the positive reaction of normal male frogs to injections of gonadotropic hormones, demonstrated by the release of mature spermatozoa in the urine of the frog.

The test for pregnancy just described does not require an understanding of the specific action of the gonadotropins (contained in pregnancy urine) that are introduced into the body of the experimental animal. Do they merely stimulate the pituitary to produce gonadotropic hormones, or act directly upon the gonadal tissue resulting in the release of sperm? There is general agreement, Chu (1946), Simpson (1944), and Oordt (1951), as to the ability of gonadotropins, as well as other specific reagents, to maintain the testicular tissue of hypophysectomized animals, even encouraging spermatogenesis in these animals. However, in reports concerning amphibia, there are no data presented to deny or confirm the ability of the pregnancy urine gonadotropins to effect spermatozoa discharge in hypophysectomized frogs.

To determine more specifically the role played by mammalian gonadotropic hormones in pregnancy tests, experimental male *Rana pipiens* from which the anterior lobe of the hypophysis had been removed, were subjected to the pregnancy test procedure.

METHODS AND MATERIALS

Mature male specimens of *Rana pipiens* were obtained from a commercial source and immediately placed in a refrigerator, simulating hibernation conditions. A total of 180 experimental animals were divided into four categories which were designated as groups A, B, C, and D (table 1).

Group A—A complete control group consisting of 90 frogs which were subdivided into two groups (A′ and A″) for the convenience of data tabulation. No surgery was performed on this group.

Group B—A partial control group consisting of 30 frogs. No surgery was performed but injections of distilled water, A.P.L. (anterior pituitary-like substance), and P.U. (a chorionic gonadotropin found in the urine of pregnant women) were administered to this group.

Group C—A partial control group consisting of 20 frogs. Incomplete surgery (anterior lobe of the pituitary left intact) was performed on each member of this group, some of which received injections of distilled water, the others receiving no injections.

Group D—An experimental group consisting of 40 frogs. Complete surgery (anterior lobe of the pituitary removed) was performed on each member of this group, some of which received injections, while others received no injections.

All injected dosages were 4 cc. per frog and consisted either of distilled water or A.P.L., or extracted P.U. The injections were administered subcutaneously by syringe into the lateral lymph sacs of the frog, using a number 23 gauge

needle. Prior to its use, the A.P.L. was diluted with distilled water until a concentration of 25 I.U. of gonadotropins to 1 cc. of distilled water was achieved. Samples of human urine were collected from one individual over a five day period (61st to 65th day of pregnancy). These samples were mixed and gonadotropic hormones extracted from them by the kaolin method.

The frogs to be treated surgically were anesthetized with ether. In this condition the animal was secured to a raised portion of an operating board by a straight pin through the tip of the upper jaw, and retractors applied deep in the mouth over the tongue and on each side of the angle of the jaws (fig. 1). Using a dental drill, a hole was bored through the mid-point of the cross formed by the parasphenoidal and transverse bone of the roof of the mouth. The dura mater in this region was carefully removed with a number 12 scalpel blade, exposing the anterior lobe of the pituitary gland (fig. 2). Preparation of the animal for experimental procedure was completed with the sewing of the flaps of the incised oral skin.

Urine samples were obtained from the experimental animal during the 2 hour post-treatment period by applying moderate finger-tip pressure on the sides of the specimen. These samples were placed on an agglutination slide and examined under a compound microscope for the presence of sperm. None of the frogs, experimental or control, was used a second time—regardless of their reaction.

DISCUSSION

While the absolute necessity of the presence of the pituitary gland to maintain life has not been entirely determined, workers in the field of endocrinology have clearly demonstrated that the removal of the gland results in extensive abnormalities. "Spermatogenesis is entirely dependent on a secretion of the anterior pituitary gland. . . . In the absence of the anterior pituitary gland follicular growth is limited or absent, and the ovaries atrophy" (Winton and Bayliss, 1949).

Sexual stimulation, in the frog, is associated with the secretion of certain hormones by the pituitary gland, one of which, the interstitial cell stimulating hormone (ICSH), is held to be directly responsible for the actual release of mature spermatozoa from the germinal epithelium of the testis. It has been established, (Wiltberger and Miller, 1948), that in the case of *Rana pipiens*, another hormone, extracted from the urine of pregnant women, also produces a gonadotropic effect and constitutes the basis for the male frog human pregnancy test.

We recognize, therefore, on one hand a hormone (ICSH) as being produced by the pituitary and acting directly upon the testis of a normal male amphibian, while on the other hand there is evidence of an identical response attributed to still another hormone (P.U.) introduced into the body of the frog. The question then poses itself as to whether this identical reaction is due to the direct action of P.U. on gonadal tissue, or an *indirect* one, wherein this hormone functions merely as an agent which stimulates the pituitary gland to produce ICSH, eventually causing the release of spermatozoa.

The results included in Table 1 may be summarized as follows:

Normal male *Rana pipiens* recently removed from hibernation,

1) respond negatively (groups A', A'' and B) when (a) untreated, or (b) injected with 4 cc. of distilled water;

EXPLANATION OF FIGURES IN PLATE

1. Position of the frog on the operating board prior to the removal of the anterior lobe of the hypophysis. Note the straight pin through the tip of the upper jaw, and retractors applied deep in the mouth over the tongue and on each side at the angle of the jaws.
2. Appearance of operation site following the removal of the thin section of bone and/or dura mater of the brain which covers the area occupied by the anterior lobe of the hypophysis. A, anterior lobe of the hypophysis.

1
2
A

2) respond positively (groups B and A") in tests for the presence of spermatozoa in the urine when (a) injected with A.P.L., or (b) injected with P.U.;
3) and when treated surgically short of pituitary removal (group C) respond negatively in tests for the presence of spermatozoa in the urine when (a) uninjected, or (b) injected with 4 cc. of distilled water;
4) and undergoing anterior lobe hypophysectomy (group D) (a) respond negatively in tests for the presence of spermatozoa in the urine when (1) uninjected, or (2) injected with 4 cc. of distilled water; (b) respond positively for the presence of spermatozoa in the urine (as long as 5 days after surgery) when (1) injected with 4 cc. of A.P.L., or (2) injected with 4 cc. of P.U.

TABLE 1

Reactions of male Rana pipiens to all conditions of the experiment

Groups	No. of Frogs	Reading	Dist. H_2O Reaction		A. P. L. Reaction		P. U. Reaction		Uninjected	
			+	−	+	−	+	−	+	−
A'	30	2 hrs.							0	30
B	30	2 hrs.	0	10	10	0	10	0		
A"	60	2 hrs.	0	20	10	0	10	0	0	20
C	20	2 hrs.	1	9					0	10
D	40	2 hrs.	0	10	10	0	10	0	0	10
		5 days					10	0		

In addition to gonadotropins, sperm release in the male *Rana pipiens* has been observed following sufficient injections of distilled water (Giltz and Miller, 1950). However, the negative reaction of groups B, C, and D to injections of 4 cc. of distilled water indicates that the positive results obtained from both normal and hypophysectomized animals following injections of 4 cc. of the gonadotropic hormones are not expressions of the animals to the quantity but rather to the quality of the liquid introduced.

Although no report of sperm emission, resulting from surgical treatment, was found in the literature, the possible association of this factor with the occurrence of false positive results could not be overlooked. Observation of the effects of partial and complete surgery, alone, and followed by injections of 4 cc. of liquid, was accomplished by subjecting smaller units of group C and D to this sequence of treatments.

The negative reactions displayed by these groups eliminate the quantity of liquid injected in combination with the effects of surgical treatment as being factors which might yield false positive results.

The reactions of the experimental specimens of group D and of the partial control group B to injections of 4 cc. of A.P.L. and P.U. are all positive. This would seem to indicate that the presence of the anterior lobe of the hypophysis is not essential in the phenomenon of sperm emission by male *Rana pipiens* following the injection of the above mentioned hormones into the body of the frog. It must be kept in mind, however, that at the time of hypophysectomy, and for a period of time thereafter, a quantity of pituitary-produced hormones may still exist in the body fluids of the experimental animals. Since all animals were tested after only a brief post-operative interval, the question might be raised

as to whether the positive reaction demonstrated by group D is an expression of the specific effect of the injected gonadotropins, or merely the result of a chain reaction in which the introduced hormones function solely in the activation of residual pituitary-produced hormones. An attempt to resolve this question was made by maintaining a portion of the animals in group D at room temperature for a post-operative period of five days. At the end of this period the animals were again treated with injections of gonadotropic hormones. The responses of the animals in this category are still positive and it can be reported that there was no apparent decrease in the intensity of the reaction after the period of five days. An equal number of normal frogs were maintained in a similar manner to determine the effects, if any, of exposure to room temperatures. There was no instance of spermatozoa emission in any of these control animals.

SUMMARY

The results of the experimentation with gonadotropins upon anterior lobe-hypophysectomized male *Rana pipiens* may be summarized briefly as follows:

1. The phenomenon of spermatozoa release, initiated by injections of mammalian gonadotropic hormones, can be accomplished in the absence of the anterior lobe of the hypophysis.
2. The specific role played by mammalian gonadotropic hormones in the pregnancy tests is believed to be a direct one upon the gonadal tissue, resulting in the emission of mature spermatozoa.

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The Biology of Paramecium. *Ralph Wichterman.* The Blakiston Company, Inc., New York, 1953. xvi+527 pp. $9.00.

This is a book which should be in the library of every teacher of zoology. Dr. Wichterman's monograph, by far the most complete account of any protozoan, signalizes the change that has come about in our conceptions of the nature of the Protozoa. Paramecium is here revealed as not merely a single protoplasmic unit but as a complex organism with a complex mode of life.

A reading of this monograph naturally suggests a comparison with the similar work of Kalmus, published in Germany in 1931. The advances made in our knowledge of the subject in the intervening 22 years are striking. Kalmus (188 pp.) listed 833 references; Wichterman (527 pp.) gives about 2000. The progress in our knowledge of the reproduction and genetics of Paramecium has been particularly great during these years. For example, all the work of Diller, of Sonneborn, and of Wichterman himself has been published since 1931.

The outstanding merits of the book far outweigh any criticisms that might be made. Two minor faults may be mentioned: the occasional typographical errors, especially in the foreign language references; and an annoying lack of consistency in the use of "**Paramecium**" and "paramecium." The latter form of the word is obviously a common noun, and in the singular should be used with an article, as in "a cat" or "an ameba."

The book is well printed and is provided with many helpful illustrations.

WENCEL J. KOSTIR.

AN ANOMALOUS APPENDAGE ON THE CARAPACE OF THE AMERICAN LOBSTER

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Over a half century ago Bateson (1894) stipulated that "to collect and codify the facts of variation is, I submit, the first duty of the naturalist;" he went on to say, ". . . a knowledge of variation lies at the root of all biological progress." Field biologists have many opportunities to observe variation of organisms, and only by placing them on record will such information, especially rarities, be available for synthesis for no one person is likely to be able to collect sufficient data through his own efforts alone. The accumulated observations of many naturalists over a period of time will eventually shed light on the mechanism of organic variations.

FIGURE 1. Lateral view of lobster showing anomalous appendage on left side of carapace.

While sampling the lobster population in Fisher's Island Sound off the coast of Connecticut in 1946, the writer discovered a unique specimen with an appendage attached to the carapace which is described and figured below. Teratological specimens of lobsters have been reported during the past century by Van Beneden (1864), Faxon (1881), Hyatt (1881), Richard (1893), Bateson (1894), Emmel (1907), Herrick (1909), Cole (1910), Jugeat (1926), Barnes et al (1932), and Templeman (1948). All of those on record involve the walking legs or rostrum. As far as known the present paper is the first record of a traumatically induced appendage growing on the carapace.

[1]This study was made while the writer was serving as Temporary Aquatic Biologist for the Connecticut State Board of Fisheries and Game and the Bingham Oceanographic Laboratory of Yale University during the summer of 1946.

By means of special traps designed for capturing all sizes of lobsters, samples were taken from Fisher's Island Sound in the summer of 1946. On August 8 a small lobster (*Homarus americanus*) which contained an anomalous appendage was trapped at a depth of 18 fathoms. It is a female specimen 17.7 cm. long with a carapace length of 6.0 cm. and weighs 158 g. The right pincher claw is missing. On the left side of the carapace, near the posterior margin, is a small appendage resembling a minature uropod (fig. 1). The exopodite measures 5 mm. by 3 mm. The endopodite measures 4 mm. by 2 mm. Each is bordered with a fringe of setae 1 to 1.5 mm. long (fig. 2). The appendage has grown from a conspicuous wound in the carapace which has healed completely. While the lobster remained alive, the appendage showed no movement. Apparently it was traumatically induced, possibly through the splitting of a nerve in the hypodermis lining the carapace. Such might have been caused by an injury at the time of molting. Herrick (1909) long ago pointed out that "Przibram has shown that in all probability monstrous

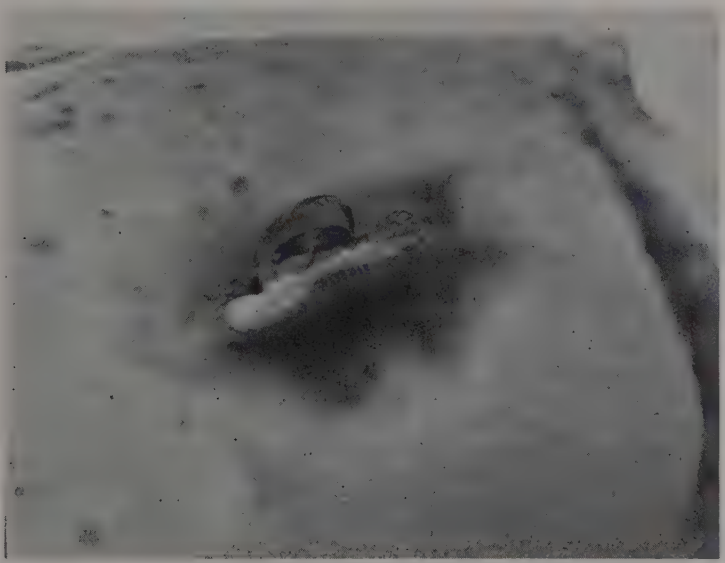

FIGURE 2. Anomalous appendage consisting of an exopodite and an endopodite growing from a wound on the carapace.

growths of every kind result from a regenerative process following upon injury. However, such growths are comparatively rare and follow only upon injury of a certain kind, or upon an injury inflicted at a certain time with respect to the molting period, or under certain conditions of the animal which are not fully understood." The appendage is heavily chitinized but has fleshy joints. Its arrangement follows Bateson's principle (1894) in that the outer branch is homologous to the exopodite while the inner branch is homologous to the endopodite. According to Emmel (1907) it is unusual to find an abnormal appendage on an immature animal.

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Mitosis, the Movement of Chromosomes in Cell Division. *Franz Schrader.* Columbia University Press, New York. Second edition, 1953. xii+170 pp. $4.00.

Despite all we know about it, mitosis is still a problem. Schrader makes this completely clear. He also creates interest in what usually is a boring description of what we do know by writing it in the form of a debate.

There are new materials and data in this second edition. I found 38 literature citations for 1951 and later years in a quick glance through the extensive bibliography. We now know that there really are fibers in spindles and this debate is satisfyingly settled. However, we now question seriously the once accepted concept that a chiasma is always evidence of an exchange of segments between pairing chromosomes and a new debate is underway. There are 3 new sections on Prophase movements, Metaphase mechanics, and Chromosome chemistry. There are 49 more pages. About one-third of this increase is due to use of a larger type face. (More publishers should do this.) The excellent and unique review of some 10 hypotheses of the causes of mitosis is still included. In the 7 years that the first edition was used as a co-textbook in one of my cytogenetics courses, I found it more stimulative of student thinking than any textbook I use in any other course. I shall certainly use this second edition for some years to come.

ELTON F. PADDOCK.

Free and Unequal. *Roger J. Williams.* University of Texas Press, Austin. 1953. xiii+177 pp. $3.50.

This book has a "message." It is particularly appropriate to review it here because the message has been presented in the pages of this journal before as an Ohio Academy of Science Presidential Address (Lindsey, A. W., Vol. 48: 169–175, 1948). I happen to know that the message was also arrived at and appreciated even before 1948 in the minds of some other geneticists. But John Doe always shies away from application of genetics to human affairs. It is therefore, noteworthy that a non-geneticist has also arrived. What is the message?

Our biological constitutions are not equal despite the Declaration of Independence, therefore freedom up to the point of not jeopardizing the freedom of others is *biologically necessary.*

The facts and arguments leading to this conclusion are presented in most charming and interesting manner. But most readers will probably find the largest reward for their investment of reading time in the latter parts of the book where the implications for everyday living are fathomed. For example, the task of the educator becomes that of *fitting*, rather than *molding* children into the social order. The children must be encouraged to develop their outstanding abilities and taught to *expect* others to be different from themselves. There will always be a statistical norm which is actually like nobody. Everybody should try to be different from it in some respect that is acceptable to society.

A task that befalls each of us is that of trying to understand the other fellow. This is different from becoming angry with and hating him, or from snobbishly dismissing him as "dumb." If the understanding is gained, the parties will be mutually better off. Such understanding will never be gained so long as we persist in the idea that all are created equal and that differences are all environmentally induced and therefore eradicable by education alone. To understand does not mean to agree. But when the cause of disagreement is understood, the possibilities of pleasant living together are greatly enhanced whether or not the disagreement persists.

In the case of differences having a predominantly genetic basis, in which something other than education will be required, we will never find the "cure" until the genetic basis is understood. It takes the truth to make us free. Read this book and you'll see!

ELTON F. PADDOCK.

STRUCTURAL AND CYTOCHEMICAL STUDIES OF THE CYTOPLASM IN THE FAMILY AMOEBIDAE[1]

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The classification and nomenclature of the cytoplasmic components and inclusions of free-living amebas were systematized by S. O. Mast and his students in a series of papers from 1926 to 1941. However, almost all of their work was done on two rather similar species, *Amoeba proteus* and *Chaos chaos*. In recent years cytochemical and quantitative physiological investigations of these two species, especially *C. chaos*, have been conducted by Holter and Andresen of the Carlsberg Laboratories in Denmark. Such information has been almost entirely lacking about other species of amebas, many of which differ strikingly in their general morphology from the two mentioned. In the present investigation, a study of the cytology and cytochemistry of three very diverse species, *Thecamoeba striata*, *Mayorella bigemma*, and *Amoeba guttula*, was undertaken, and *Amoeba proteus* and *Chaos chaos* were studied at the same time for purposes of comparison.

Along with the classical cytological techniques, including vital staining, a cytochemical approach has been employed for a better understanding of the nature of the cell components through a knowledge of their chemical composition. Since the majority of present histochemical methods are designed for use with metazoan tissues, special techniques had to be devised for applying these tests to protozoa.

MATERIALS AND METHODS

The Species Studied

Amoeba proteus (Pallas, 1766) Leidy, 1878. Cultures of *Amoeba proteus* have been maintained continuously in the Protozoology Laboratory at The Ohio State University for many years. The culture method, using *Saprolegnia* sp. growing on rice grains, with *Chilomonas paramecium* as the chief food organism, has been described in detail by Handy (1947).

Many synonyms exist for *Amoeba proteus*. (Mast and Johnson, 1931). However, the only other name used today is *Chaos diffluens*, proposed by Schaeffer in 1926. *Amoeba proteus* can be distinguished from two similar species, *Amoeba dubia* and *Amoeba discoides*, by the presence of ridges on one or two of its larger pseudopodia. Also *Amoeba proteus* measures 600 or more microns when elongated, whereas *Amoeba discoides* and *Amoeba dubia* rarely measure more than 400 microns (Kudo, 1946; Jahn and Jahn, 1949).

The cytoplasm of *Amoeba proteus* has undoubtedly been studied more often than that of any other species of ameba. The nomenclature of the various inclusions and structures of the cytoplasm here employed is that proposed by Mast (1926) in his first paper on the structure of this species.

Chaos chaos Linnaeus, 1758. This so-called giant ameba has, along with *Amoeba proteus*, been maintained continuously in the Protozoology Laboratory of The Ohio State University for many years. The culture method employed is similar to that for *Amoeba proteus*. Except for its tremendous size (1–3 mm.) and multinucleate condition, *Chaos chaos* is rather similar to *Amoeba proteus*.

The giant ameba has been designated by several scientific names, the most common being *Chaos chaos* Linnaeus and *Pelomyxa carolinensis* Wilson.

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[1]From a dissertation submitted to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Thecamoeba striata (Penard, 1890) Schaeffer, 1926. *Thecamoeba striata* is characterized by the presence of a pellicle which has prominent longitudinal ridges or folds on the upper surface during locomotion. In one respect it differs rather strikingly from all other amebas, both pelliculate and non-pelliculate. It contains a contractile vacuole of irregular and changing shape. Most individuals have a second contractile vacuole which is always spherical.

Thecamoeba striata measures up to 120 μ during locomotion. It is limax shaped, seldom forming any pseudopodia; the whole organism advances with an anterior hyaline cap making up as much as one-third of the entire animal. This anterior hyaline cap is always indicative of the direction of locomotion. A smaller form of *Thecamoeba striata* was described by Penard (1902) and by Pappas (1948). Most of the observations recorded in the present paper were made on the larger of the two varieties.

Clone cultures of *Thecamoeba striata* have been maintained in Reynolds' (1924) strained hay infusion. Ten grams of timothy hay (*Phleum pratense*) are boiled in 250 ml. of distilled water for 15 min. The broth is then strained through cheese cloth and diluted with 2500 ml. of distilled water. Cultures of *Chlamydomonas apiculata* in its passive stage are used as the food organism for the ameba. The cultures are prepared in Syracuse watch glasses.

Mayorella bigemma (Schaeffer, 1918) Schaeffer, 1926. *Mayorella bigemma* is characterized by the presence of small conical pseudopodia which form continuously along the anterior edge and on the upper surface while the animal is in locomotion. These pseudopodia do not determine the direction of locomotion, but are carried along by the ameba as a whole. They are not static, but rather in continuous extension and retraction during locomotion. Both the anterior margin of the ameba and the conical pseudopodia are composed of clear, hyaline cytoplasm. Individuals of *Mayorella bigemma* vary from 60–200 μ in length during locomotion.

Clone cultures of *Mayorella bigemma* were established under the same conditions as those for *Thecamoeba striata*, with *Chlamydomonas apiculata* as the food organism in strained hay infusion. Also *Ochromonas sp.*, *Euglena gracilis*, and bacteria have been used successfully as food organisms in strained hay infusion. The cultures were maintained in short stender dishes. Detailed accounts of culture methods and behavior of *Mayorella bigemma* have been given by Lotze (1934, 1937).

Amoeba guttula Dujardin, 1841. *Amoeba guttula* was found thriving in the top scum formation of hay infusion cultures a week or two after inoculation. The pH of the top scum was 5.0–5.3. When the pH became alkaline, the scum tended to sink to the bottom of the culture. At this time *Amoeba guttula* disappeared completely. Bacteria are the chief food of this organism.

Amoeba guttula is the smallest of the five amebas studied, measuring 20–25 μ during locomotion. This ameba has lobose pseudopodia which are extremely short and composed entirely of hyaline cytoplasm. Because they are both lobose and short, they often appear almost circular. Each pseudopod appears and develops with characteristic abruptness. At the anterior end of the ameba about one-third to one-half of the cytoplasm is hyaline.

Preparation of Amebas

General. The most useful method of investigating the cytoplasm of amebas is undoubtedly by study of the living specimens. Vital stains and phase contrast microscopy have both proven extremely helpful in such study.

However, one cannot avoid the use of killed and fixed material, since most stains and chemicals which are of value for the study of cellular constituents kill the organism. Some reagents, such as Lugol's solution and methyl green, serve as both killing and fixing agents, and are usually employed with simple mounts. However, for most cytochemical methods, more complicated procedures are necessary, including the use of various special reagents. These will be described on a later page.

Many methods have been described for the preliminary handling in preparation for fixing, staining, etc., of protozoa. (See McClung's Handbook of Microscopical Technique, 3rd ed. p. 441 *et seq.*) Most of these methods were tried but with indifferent success and were abandoned in favor of the parlodion trap technique described below.

The Parlodion Trap Technique. The parlodion trap method used in this study was developed by Concannon (1951) for handling small nematodes. With slight modifications, this method was found to be very satisfactory for handling amebas in preparation for subsequent staining, mounting, etc.

The parlodion solution used consists of 3½ g. of parlodion (highly purified cellulose nitrate) dissolved in 100 ml. of absolute alcohol and 100 ml. of ether. The solution should be kept under refrigeration.

The trap itself is a loop made of lacquer coated wire (about #30 B & S gauge) by taking a piece about three inches long, wrapping it once around some cylindrical object, such as glass tubing, and then twisting the free ends together. The circlet is designated as the trap, and the twisted part the handle. The trap and handle are flattened so that both are in the same plane (fig. 1).

FIGURE 1. Diagram of parlodion trap for collecting amebas.

FIGURE 2. Diagram showing position of trap in reagent and staining dishes.

The procedure is as follows. A drop of medium containing amebas is put on a perfectly clean slide and allowed to stand until only a thin wet film remains. A drop or two of absolute alcohol is then added to the middle of the film, which forces the water to the film's periphery. The alcohol fixes the organisms and most of them remain in their original location. The remaining water is then removed with filter paper. If some other fixing reagent is used before the treatment with absolute alcohol, more of the amebas are lost for they do not adhere as well to the glass and are thus forced to the periphery of the film with the water.

When almost all of the absolute alcohol has evaporated from the film, the trap is placed on the slide so that most or all of the film containing the amebas will be inside the loop. One or two drops of parlodion solution are then dropped on the slide within the loop. Care should be taken to see that too much of the solution is not added to the loop, since the parlodion would then be difficult to de-stain. When the parlodion is almost dry, the slide should be tipped so that excess solution will run down the handle from the loop. In this way, a very thin membrane in which the amebas are embedded remains in the loop.

The slide, with the trap in place, is then either flooded or gently immersed in water while being kept horizontal. This is a very important step, as water must penetrate between the parlodion and the glass slide to prevent adhesion between them. After a few minutes, the trap may be removed from the slide by grasping the wire handle with forceps and lifting gently upwards. If the effort has been successful, a very thin membrane of parlodion is left within the wire loop, and in this membrane the amebas are embedded. In a certain percentage of trials, the parlodion film ruptures, but approximately 70–80 percent of the attempts are successful. The diameter of the wire loop should not exceed 12–13 mm., since the membrane ruptures easily on larger loops.

For the subsequent steps (staining, etc.) it is desirable that shallow reagent and staining dishes be used in order that the wire handle of the trap should not be completely immersed in the solutions (fig. 2). If several traps are to be treated

identically, the end of the handle of each may be bent into a hook. Then the traps can be hung from glass rods or thick wire, etc., and in this manner transferred from solution to solution.

The parlodion membrane will take up stain along with the amebas embedded in it, but will lose the stain much more readily than the amebas during washing or passage through alcohol.

Parlodion is soluble in absolute alcohol, so that it is necessary that the final dehydration in the preparation of permanent slides be accomplished by the use of 25 ml. melted carbolic acid crystals to 75 ml. of xylene. When the trap is first placed in this solution from 95 percent alcohol, the parlodion membrane will appear milky, but it usually becomes clear within a few minutes. In any event, the trap should be left in the carbol-xylol long enough for the membrane to appear completely transparent, for it is not until then that the water has been completely removed. The trap is then transferred to xylene.

After a few minutes the trap is taken out of xylene, drained to remove the excess, and placed onto a drop of mounting medium on a clean slide. (Any xylene-soluble mounting medium is satisfactory.) A drop of the mounting medium is then placed on top of the parlodion membrane. The latter is then freed from the sides of the wire loop with a sharp dissecting needle or scalpel, and a very thin coverslip(#0 preferred) is placed on the mounting medium.

The parlodion trap technique is not suitable for some cytochemical tests, because both free lipids and free carbohydrates are removed by the organic solvents. However, for reactions such as Feulgen's and Millon's and for water insoluble polysaccharides visualized by the PAS reaction, material prepared by the parlodion trap method is quite adequate.

Cytochemical Methods

Cytochemistry is concerned with the localization of chemical entities in living animal and plant cells. Lison (1936) first systematized the criteria by which one must evaluate cytochemical technique, and the reader is referred to his discussion of the subject.

Since almost all of the cytochemical methods now employed were originally designed for use with metazoan tissues, it was necessary to devise modifications in applying these tests to protozoa. Such modifications are incorporated into the descriptions of these methods in the following pages.

Reactions Indicating Proteins

Millon's reaction for tyrosine. The cytochemical adaptation of Millon's reaction was accomplished by Bensley and Gersh (1933). The procedure, as outlined in Lison (1936), is modified for this study as follows:

1. Amebas mounted in a parlodion trap are placed in Millon's reagent (at room temperature) for about 5 hr. or until maximum color develops.
2. The trap is then immediately placed in 1 percent nitric acid solution, and left there for 2–3 min.
3. The trap is placed on a slide, and 2–4 drops of absolute alcohol are dropped on the parlodion film, which dissolves almost immediately. The trap is removed and the cover-slip is placed on the alcohol, which now contains all the amebas that were in the film. More absolute alcohol is added at the edge of the cover-slip as needed.

Only temporary mounts are usually attempted with this procedure since the color of the preparation fades within a few days.

Results. A rose or brick color indicates the presence of tyrosine-containing protein in the cell. The coloration is due to the particular aromatic radical found in tyrosine. However, Lison (1936) points out that a certain number of non-protein phenol compounds may also give this reaction. Their distribution in cells, however, is quite limited.

Arginine. Baker's (1947) modification of the Sakaguchi test for arginine was employed. Reagents and procedures were modified in the present study as follows:

Reagents. 1. Absolute alcohol as a fixing agent.

2. Parlodion, 3.5 percent, in a mixture of absolute alcohol and ether in equal volumes.

3. NaOH, 1 percent, aqueous.

4. α-naphthol, 1 percent, in 70 percent alcohol.

5. Hypochlorite solution (2 volumes sodium hypochlorite and 1 volume of 0.05 N NaOH).

6. A mixture of 3 volumes of pyridine with 1 volume of chloroform.

Procedure. 1. Amebas are mounted in a parlodion trap. The trap is then placed in distilled water.

2. Then 2 ml. of 1 percent NaOH are put into an old-fashioned watch glass and 2 drops of α-naphthol solution and 4 drops of hypochlorite solution are added.

3. The parlodion trap is now placed in a depression slide. The mixture just described is then gently shaken and quickly added to the parlodion-embedded amebas on the trap and allowed to remain for about 15 min.

4. The trap is then transferred to a flat slide and pyridine-chloroform mixture is added. This dissolves the film so that the trap may be removed at once, and a cover-slip is placed over the mixture with its contained amebas. More of this mixture is added at the edge of the cover-slip as needed to make up for evaporation.

Results. A pink or red color indicates the presence of arginine (free or combined) or some other positively-reacting guanidine derivative in cells. The test is based on the development of a red color by arginine when α-naphthol and hypochlorite react with it in an alkaline medium.

Feulgen nucleal reaction. This reaction makes possible the visualization of desoxyribonucleic acid (DNA). Indeed, the Feulgen reaction is considered to be one of the most specific cytochemical reactions (Stowell, 1946). The reagents and procedures were modified from those outlined by Glick (1949).

Reagents. 1. Fixing reagent—absolute alcohol or formaldehyde-calcium (10 ml. of full strength formalin, 10 ml. of 10 percent calcium chloride (anhydrous) and 80 ml. of distilled water).

2. HCl, 1 N.

3. Coleman (1938) preparation of Schiff's reagent.

4. Sulphurous acid solution—add 30 ml. of 1 M sodium bisulfate solution and 30 ml. dilute HCl to 600 ml. of tap water.

Procedure. 1. Amebas fixed in either absolute alcohol or formaldehyde-calcium are mounted in parlodion.

2. Parlodion traps containing the amebas are then placed for a minute or two in HCl solution at room temperature.

3. They are then transferred to HCl solution at 60° C for 4–5 min.

4. Then they are treated with Schiff's reagent for 1½–3 hr.

5. The trap is then passed through three separate baths of the sulphurous acid solution and left in each for 1–2 min., agitating frequently.

6. Then the trap is washed for about 5 min. in tap water.

7. The trap is then dehydrated and cleared as has been described for the parlodion trap technique, and mounted in balsam.

Results. A purple color indicates the presence of desoxyribonucleic acid. Acid hydrolysis removes the purine bases and exposes free aldehyde radicals which stain purple when treated with fuchsin-sulfurous acid of Schiff's reagent. The sulphurous acid solution removes the excess Schiff's reagent from the cell.

Detection of ribonucleic acid. Visualization of ribonucleic acid (RNA) in the cell is accomplished cytochemically by the use of subtractive methods, these being either depolymerization of RNA with ribonuclease or extraction with perchloric

acid as compared with non-treated controls. Since nucleoproteins are basophilic, basic dyes such as toluidine blue O will reveal their presence in the cell.

The ribonuclease technique. The technique used in this study was modified from that recommended by Stowell and Zorzoli (1947).

Reagents. 1. Fixing reagent—formaldehyde.

2. Ribonuclease (Worthington Biochemical Company, Freehold, New Jersey) 0.1 mg. per ml. of buffer (McIlvaine's citric acid-disodium phosphate mixture buffered at pH 6.5).

3. Toluidine blue O—0.5 percent in distilled water.

Procedure. 1. Amebas, fixed in formaldehyde, are embedded in parlodion, using the parlodion trap technique.

2. One group of amebas is then put into the ribonuclease solution at 50° C while the other is put into plain McIlvaine buffer at 50° C. Both are kept under these conditions for at least 3 hr.

3. Both groups are quickly washed in separate dishes with distilled water.

4. Then in separate staining dishes, both groups are treated for a few minutes with toluidine blue O. After another quick rinse in distilled water, the amebas are mounted in distilled water.

Results. The ribonuclease-treated group is compared with the non-treated group, on the basis of the presence and distribution of basophilic substances. The absence of basophilia in the treated amebas as compared to the non-treated controls indicates RNA.

Perchloric acid technique. The method used here was modified from those of Seshacher and Flick (1949) and Erickson, Sax, and Ogur (1949).

Reagents. 1. Fixing reagent—acetic acid alcohol mixture (1 volume acetic acid to 3 volumes of 95 percent alcohol).

2. Perchloric acid ($HClO_4$)—5 percent in distilled water.

3. Toluidine blue O—0.5 percent in distilled water.

Procedure. 1. Amebas fixed in acetic acid alcohol mixture are embedded in parlodion, using the parlodion trap technique.

2. One group of amebas is then put into the perchloric acid solution at 70° C while the other is put into plain distilled water also at 70° C. Both groups are kept under these conditions for about 30 min.

3. Both groups are quickly washed in separate dishes with distilled water.

4. Then, in separate staining dishes, both groups are treated for a few minutes with toluidine blue O. After another quick rinse in distilled water, the amebas are mounted in distilled water.

Results. The perchloric acid treated group is compared with the non-treated group as to the presence and distribution of basophilic substances. The absence of basophilia in the treated amebas as compared to the non-treated controls indicates the presence of RNA.

Carbohydrate Reactions

Lugol's solution (4 g. iodine, 6 g. potassium iodide per 100 ml. of solution) was used as fixing reagent and as a stain for starch.

Periodic acid-Schiff (PAS) reaction for polysaccharides. McManus (1946) and Hotchkiss (1948) have formulated a cytochemical reaction for the visualization of polysaccharides such as glycogen, mucin, mucoproteins and presumably hyaluronic acid and chitin. The method of Hotchkiss was modified for this study.

Reagents. 1. Fixing reagent—absolute alcohol.

2. Periodic acid solution (H_2IO_6)—0.9 percent in distilled water. Take 45 ml. of this concentration and add 5 ml. M/5 sodium acetate.

3. Iodide-thiosulfate solution. Dissolve 1 g. potassium iodide and 1 g. sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in 20 ml. of distilled water and add with stirring

30 ml. of alcohol followed by 0.5 ml. of 2 N HCl. A sulfur precipitate forms and settles out slowly, although the solution may be used immediately.

4. Schiff's reagent. (For preparation, see Feulgen nucleal reaction.)

5. Sulfite wash solution. Add 0.5 ml. concentrated HCl and 2 ml. of 10 percent potassium metabisulfite to 50 ml. of distilled water.

Procedure. 1. Amebas, fixed in absolute alcohol, are embedded in parlodion, using the parlodion trap technique.

2. The amebas, embedded in parlodion, are placed in the periodic acid solution for about 10 min.

3. Washed with water, they are then transferred to the iodide-thiosulfate solution for about 10 min.

4. Again they are washed with water and placed in Schiff's reagent for about 30–60 min.

5. They are then rinsed in the sulfite wash solution for a few minutes.

6. The amebas are then dehydrated and mounted in balsam in the usual manner for the parlodion trap technique.

Results. A violet color indicates the presence of polysaccharides. Polysaccharides are oxidized under mild conditions by periodic acid to polyaldehydes. Then the iodide thiosulfate solution removes the periodic acid from the cells. The polyaldehydes are then colored by Schiff's reagent. Excess Schiff's reagent is then removed by the sulfite wash solution. The pentoses of nucleic acid are so affected by periodic acid that they will not react.

Lipid Reactions

Lipids may be characterized by their sudanophilic properties, i.e., they become colored by Sudan reagents. The Sudan substances are not dyes because they are not ionized. They color lipids by dissolving in them. (Lison, 1936; Cain, 1950).

Sudan III, IV and black B were prepared as saturated solutions in 70 percent alcohol and filtered twice. Sudan black B, the most powerful of the Sudan reagents, was also prepared in ethylene glycol as suggested by Chiffelle and Putt (1951). According to Cain (1950), coloration with Sudan black B depends ultimately not on unsaturation but on the physical state of the object tested.

A 1 percent solution of nile blue sulfate was used on amebas which usually were fixed previously in formalin. Nile blue sulfate has been used to distinguish neutral fats (triglycerides) from fatty acids, the former being dyed red or pink, the latter blue. Lison (1936), after a detailed study, concluded that the red coloration was characteristic of lipids in general and the blue was merely that of a basic dye and therefore totally unspecific. Cain (1947) maintains that if a substance known to be a lipid colors red with nile blue, it consists of neutral lipids; if it colors blue, it may contain these, but acidic lipids (fatty acids, phospholipids and others) may also be present.

The specificity of the extended procedure required by Baker's (1946, 1947) acid hematin test for the recognition of phospholipids is based on empirical criteria. In this study it was not possible to carry out each step of the test exactly as outlined by Baker. Unless new empirical data were to be collected, results which would have been obtained by modifying the original test would have had no cytochemical meaning.

The Schultz method for the demonstration of cholesterol was used as outlined by Lison (1936). After the amebas have been placed in a 2.5 percent iron alum solution at 37° C. for about 8 hr., they are transferred to a slide. Then they are treated with a few drops of acetic-sulphuric mixture (1 part glacial acetic acid in 1 part concentrated sulfuric acid). If a blue-green color develops, then cholesterol is present. If the color does not develop then no conclusion can be drawn, since negative results in the Schultz test do not mean that cholesterol is absent. (Lison, 1936.) The mordant transforms cholesterol to oxycholesterol,

which in turn becomes a colored substance when acted upon by the acetic-sulfuric mixture.

Plasmal reaction. Feulgen and Voit (1924) have described the demonstration of acetal lipids in the cell. From these acetal lipids ("plasmalogen"), the "plasmal" (free aldehyde) is unmasked. The procedure employed in this study for the visualization of the "plasmal" is that suggested by Hayes (1949).

Reagents. 1. $HgCl_2$, 1 percent aqueous solution.

2. Schiff's reagent. (For the preparation, see Feulgen nucleal reaction.)

3. Sulfurous acid wash solution. (For preparation, see Feulgen nucleal reaction.)

Procedure. 1. One group of amebas is placed in 1 percent $HgCl_2$ in a depression slide for about 3–5 min.

2. This group and a control group are transferred into Schiff's reagent in two separate depression slides and left for about 10 min. The depression slides must be covered during this period.

3. Both groups are then transferred into depression slides containing the sulfurous acid wash solution, and left for 2 min. This step is repeated three times.

4. The amebas are then transferred into depression slides containing distilled water.

5. Temporary mounts are then prepared in distilled water.

Results. The presence of violet fuchsin color indicates acetal lipids. According to Feulgen and Voit (1924), the brief action of $HgCl_2$ on the acetal bond of the bound plasmalogen splits it into a fatty aldehyde (plasmal) and glycero-phosphoro-ethanolamine. The plasmal, being an aldehyde, is then readily colored by Schiff's reagent. Hayes (1949) maintains that the plasmal reaction is highly specific for acetal lipids alone, if the time for the action of the $HgCl_2$ is limited to insure that neither acid hydrolysis nor oxidation takes place, and if controls are carefully checked. As far as is known the only acetal lipids which have been demonstrated as present in cells are phospholopids. However, other, non-phospholipid acetals might exist.

Other Methods, Reagents, and Stains

Sulfhydryl Test. The nitroprusside test for sulfhydryl groups as outlined by Chalkley (1937) was employed. One drop of 20 percent zinc acetate was placed on a clean depression slide. By means of a micropipette, amebas were transferred into the zinc acetate solution. Care was taken so that only a minimum of culture medium was transferred with the amebas. One drop of a 1 percent sodium nitroprusside solution was then added. A rose red color results where sulfhydryl is present. Since the color fades in a few hours, observations on the amebas in the depression slide must be made immediately. The nitroprusside test has been severely criticized by Lison (1936) and Hammett and Chapman (1939) as being unreliable as a quantitative reaction and also because the resulting coloration may diffuse throughout the cell. Bennett (1948) has developed a more accurate method for the cytochemical determination of sulfhydryl, employing a compound which was synthesized specifically for this purpose. The compound was not available, however, for this study.

Miscellaneous. A 2 percent solution of osmium tetroxide (0.5 g. osmic acid crystals in 25 ml. distilled water) was employed as a fixing reagent. Exposure to the OsO_4 vapors of this solution for 30 sec. to 2 min. was quite satisfactory.

Commercial formalin (40 percent formaldehyde) vapors were employed for fixing, as well as 10 percent solutions of formalin. Formalin-calcium solution (10 ml. full strength formalin, 10 ml. calcium chloride 10 percent, anhydrous, and 80 ml. distilled water) was also used. Champy's fluid (70 ml. of 3 percent potassium bichromate, 70 ml. of 1 percent chromic acid, 40 ml. of 2 percent osmium tetroxide) was used as a fixing agent for mitochondria. Transeau's fixative (H_2O—6 parts, 95 percent alcohol—3 parts, formalin—1 part) was used as a

general fixing reagent. Schaudinn's fluid (saturated aqueous solution $HgCl_2$—2 parts, 95 percent alcohol—1 part) was also used as a routine fixing agent.

A 0.02 percent solution of ruthenium red (ruthenium oxychloride, ammoniated, 1 : 5,000) was employed. Neutral red was used in the following concentrations: 1 : 75,000 (0.00133 percent), 1 : 100,000 (0.001 percent), and 1 : 150,000 (0.00077 percent). Janus green B was used in a concentration of 1 : 100,000 (0.001 percent). A 0.2 percent solution of methyl green was prepared in 1 percent acetic acid. Aniline fuchsin for staining mitochondria was prepared from 10 g. acid fuchsin in 100 ml. of aniline water (8 ml. aniline oil in 180 ml. of distilled water). Of the hematoxylin stains, Delafield's and Heidenhain's iron hematoxylin were used. A 2 percent iron alum solution was used as a mordant when staining with Heidenhain's iron hematoxylin. Toluidine blue O was prepared as a 0.5 percent solution in distilled water as recommended by Lison (1936). A stock solution of 3 percent H_2O_2 was also used.

Doyle's (1933) preparation for the simultaneous demonstration of fat and starch was used.

Microscopes and Accessories

A Spencer (American Optical Company) binocular research compound microscope with apochromatic objectives (16, 4, and 2 mm.) and compensating oculars (10, 15, and 20×) was used throughout this study. A phase-difference microscope (American Optical Company) equipped with a 97× B minus contrast (low) objective as well as 10, 43, and 97× dark contrast (medium) objectives was also used. A stereoscopic binocular microscope (American Optical Company) was used as an aid in manipulating and transferring amebas. A polarizing microscope (American Optical Company) was used to determine optical activity of crystals.

Measurements were made with the aid of ocular micrometers. Each microscope was calibrated for use with a specific ocular micrometer. All drawings were made with the aid of a camera lucida.

RESULTS

The Cell Membrane

The cell membrane or outermost limiting layer is a seemingly unspecialized plasma membrane in *Amoeba guttula* and *Mayorella bigemma*. In the genus *Thecamoeba*, there is formed an outer relatively thick covering called a pellicle. Howland (1924c) removed the pellicle from *Thecamoeba verrucosa*, leaving the plasma membrane intact. Apparently intermediate between a thickened pellicle and an unspecialized plasma membrane is the outer covering, the plasmalemma, found in *Amoeba proteus* and *Chaos chaos*.

Thecamoeba striata is characterized by the presence of a number of delicate longitudinal lines (usually 4 to 6) on the surface. These longitudinal lines are present during locomotion or whenever the ameba is elongate. When it rounds up, there are many folds and indentations on the surface. The width of the longitudinal lines is about 0.75 μ. The thickness of the pellicle is around 0.33 μ if we assume that each longitudinal fold is composed of two layers of pellicle with little, if any, material between them. Measurements of the pellicle made at the edge of the ameba, whether living or fixed, were approximately the same.

Unless an extreme amount of pressure is applied to the coverslip, *Thecamoeba striata* will not rupture even in the event of evaporation of the surrounding medium. The pellicle does not stain with neutral red, nor does it hamper the entrance of this stain into the cytoplasm to any great degree. The time required for given concentrations (1 : 75,000, 1 : 100,000 and 1 : 150,000) of neutral red to appear within the cytoplasm of this ameba is about the same as in the non-pelliculate amebas studied.

When *Amoeba proteus* and *Chaos chaos* are active and have extended pseudopodia, the plasmalemma is generally smooth and without folds. Wrinkling

of the plasmalemma occurs only when the cells are rounded up. The thickness of the plasmalemma was found to be about 0.25 μ. It should be noted that the plasmalemma of these amebas ruptures much more easily than the pellicle of *Thecamoeba striata*.

In the other amebas studied, *Amoeba guttula* and *Mayorella bigemma*, the thickness of the cell membrane was not ascertained since it is extremely thin and much less than 0.25 μ.

Cytochemistry of the cell membrane. The cytochemical reactions hereinafter described were very easily visualized in the cell membranes of all the amebas studied, both pelliculate and non-pelliculate. In the extremely thin plasma membranes of *Mayorella bigemma* and *Amoeba guttula*, the results were not as pronounced as in the other species, but were definite nevertheless.

The cell membrane in all the amebas stained red with 1 : 5000 ruthenium red (ruthenium oxychloride, ammoniated). This metallic pigment has been employed in microscopic work as a test for pectin in plant cells (Bonner, 1936, 1946, 1950). Its specificity for pectin alone, however, is greatly in doubt. Hence no definite conclusions as to the chemical nature of the cell membrane can be drawn from the use of ruthenian red.

The cell membrane appeared violet after being treated by the PAS reaction. As was stated earlier, this reaction involves the oxidation of adjacent hydroxyl groups to aldehydes with Schiff's reagent. Thus the presence of the violet fuchsin color indicated that the pellicle of *Thecamoeba striata* as well as the cell membranes of all the other species contain polysaccharides.

Since non-figured (free) glycogen is water-soluble to some degree, and of course the cell membrane is not, it cannot be present as an important constituent of the cell membrane. However, glycogen could be present as a figured substance, i.e., combined with or bound to some other substance and thus not necessarily water soluble. By the application of saliva, the pellicle was found to be ptyalin-resistant, thus showing that it does not contain figured glycogen.

The Millon reaction (Bensley and Gersh, 1933) for tyrosine and the Sakaguchi test (Baker, 1947) for arginine were employed. Since the vast majority of proteins contain tyrosine and arginine, these two cytochemical tests are actually good protein indicators. The cell membrane stained red with both tests, indicating the presence of protein. Thus the cell membrane of all the amebas tested contains protein and polysaccharides. The saliva-resistant, PAS positive, protein-combined carbohydrates belong to the muco-polysaccharide group. A muco-polysaccharide (mucin) is a compound containing protein and polysaccharide in which the polysaccharide is predominant.

Some basic dyes stain certain cell components the same color as that of the dye itself, but stain certain other elements with a different color. This is called metachromatic staining. It has been explained (Lison, 1936) that muco-polysaccharides which stain metachromatically, with toluidine blue, for instance, (usually pink or red) are acid polysaccharides and usually contain sulfate. Hyaluronic acid is the common acid polysaccharide found to be present intercellularly in metazoan tissues, although it does not contain sulfate. The cell membrane in the amebas was found not to be metachromatic. That is, it stained blue with toluidine blue, the same color as the dye. As a further check, several amebas were treated with hyaluronidase before staining with toluidine blue. Those treated with hyaluronidase did not stain any differently than the non-treated controls. Therefore it may be concluded from these tests that the cell membrane is composed in part of a neutral mucopolysaccharide or mucin.

Cytoplasmic Components and Inclusions

The Alpha Granules. The alpha granules are rather abundantly represented in all the amebas studied. In the unstained living cells they are easily seen with the phase-difference microscope. The size of these granules, about 0.25 μ, appears

to be the same in all the species studied. Due to the fact that the staining reactions were inconclusive, nothing could be ascertained concerning the nature of these granules.

The Beta Granules. The beta granules in the five species of amebas studied are very similar in size and shape. They are predominantly spherical and measure about 1 μ in diameter. Although Mast and Doyle (1935a) claim that the beta granules change their shape in *Amoeba proteus*, no evidence for this was obtained in the present study. However, in *Thecamoeba striata* (and in no other species studied) coalescence of beta granules has been observed several times both in stained and unstained amebas. The coalesced granules appear as homogeneous spheres, measuring up to 2.6 μ in diameter. Six to ten such spheres have been found in some individuals. Clumps of beta granules have also been observed in this species, but these granules retain their individual shapes in the clump. No direct observations were made as to whether this clumping of granules precedes coalescence; but this seems to be probable. Coalescence and clumping of beta granules have not been reported previously in amebas. Bourne (1951) reports that clumping and coalescence of mitochondria in certain mammalian cells often occurs in cases of scurvy. The significance of this phenomenon in *Thecamoeba striata* is not known.

The beta granules may be considered under two headings, those that surround the contractile vacuole and those that do not. The observations made in this study corroborate the conclusions of Mast and of Andresen that this division is purely an artificial one, since the only difference in the granules surrounding the contractile vacuole is that they are more or less flattened as they lie on the surface of this vacuole. The staining qualities, plasticity, and composition appear to be the same in all the beta granules, regardless of their distribution in the cell.

Janus green B has been found to be a specific stain for beta granules. (Mast and Doyle, 1935a, 1935b; Andresen, 1942.) Cytologists have long considered Janus green B a specific stain for metazoan mitochondria. (McClung, Handbook of Microscopical Technique, 3rd ed., p. 140.) When used in optimum concentration (1 : 100,000) the beta granules, and no other structures, appear distinctly green after about 3 or 4 hr. Mast and Doyle (1935a) declared that in *Amoeba proteus* only the outer layer of the beta granules stained with Janus green B, indicating a differentiated surface layer. In the present investigation the same results were at first obtained with *Amoeba proteus* and *Thecamoeba striata*. However, when the amebas were left in the Janus green solution for more than 24 hr., the granules appeared homogeneous. Observations made with the phase-difference microscope on unstained amebas also showed no differentiation of parts.

Mast and Doyle (1935a) applied classical metazoan mitochondrial staining methods to *Amoeba proteus* and found that the beta granules behaved like the mitochondria of metazoa. Andresen (1942) used aniline fuchsin after Champy-Kull fixation and successfully stained the beta granules of *Chaos chaos*. These methods were repeated in the present study and similar results were obtained. Since Janus green B and the classical mitochondrial staining methods definitely stain these granules, it seems very probable that the beta granules are actually the mitochondria of amebas.

Mast and Doyle (1935b) state that the beta granules are much more numerous at the surface of the food vacuoles during the initiation of digestion and toward the end of this process. Today, observations on this point are made much easier and more definite by the use of a phase-difference microscope, which was not available to Mast and Doyle. Such observations at different phases of digestion revealed no beta granules definitely associated with food vacuoles at any time.

Cytochemistry of beta granules. The beta granules gave a positive reaction when treated with Millon's reagent for tyrosine and Sakaguchi test for arginine. In order to test for the presence of lipids in the beta granules, amebas were treated

with Sudan III and also Sudan IV in 70 percent alcohol. The results were negative. When Sudan black B was employed, however, the beta granules did show a faint bluish-black color. The affinity of the Sudan black B for the beta granules was greatly enhanced when ethylene glycol (Chiffelle and Putt, 1951) was used as a solvent instead of 70 percent alcohol. The PAS reaction for polysaccharides gave negative results. Ribonuclease and perchloric acid techniques for the presence of ribonucleic acid also gave negative results. It seems, therefore, that if ribonucleic acid is present in these granules it is in minute quantities. Using the nitroprusside test, negative results were also obtained for sulfhydryl groups. The cytochemical tests described above suggest that the beta granules of the amebas studied are lipid and protein in composition, as are metazoan mitochondria.

The Spherical Refractive Bodies. Numerous spherical refractive bodies varying in size and number were found in three of the five species studied, (*Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*). They vary from 1.4 to 7 μ in diameter. At times it was estimated that as many as 200 were present in *Chaos chaos*, while *Amoeba proteus* was found to have as many as 40 and occasionally as few as 4 of these bodies. The number in *Thecamoeba striata* varies from as many as 20 to as few as 6.

In *Amoeba proteus* and *Thecamoeba striata* these spherical refractive bodies appear homogeneous in unstained individuals, and stain red, vitally, with neutral red. However, when various concentrations of neutral red (1 : 75,000, 1 : 100,000 and 1 : 150,000) were used, it was found that only the outer cortical layer of the larger bodies was stained. In many of the smallest of these bodies (those measuring 1.4–1.8 μ) no such differentiation between inner and outer portions was shown, and neutral red stained them homogeneously in the living ameba. In the larger spherical refractive bodies, the differentiation of cortical and inner regions was also observed in untreated living amebas with the aid of the phase-difference microscope.

The spherical refractive bodies in *Chaos chaos* did not in most cases stain vitally with neutral red. This is in accord with the observations of Andresen (1942). In one or two living individuals, however, a few of the larger bodies did stain. In every other respect, the spherical refractive bodies were found to be alike qualitatively in the three species of amebas in which they were present.

Osmium tetroxide vapors blacken the outer portion of the spherical refractive bodies. Subsequent treatment with 3 percent hydrogen peroxide did not bleach them. The outer portion also stained a faint grey-blue with Sudan black B, indicating the presence of lipid material. Both Millon's reagent for tyrosine and the Sakaguchi test (Baker's 1947 modification) for arginine gave a pink coloration of the same intensity as the cytoplasmic ground substance indicating the presence of protein in the cortex of the refractive bodies. Mast and Doyle (1935a) claim that between the inner and outer portion of these refractive bodies a layer of carbohydrate is present. However, in the present study only negative results were obtained when the PAS reaction was used to test for the presence of polysaccharides in them, and Lugol's solution did not demonstrate any starch.

Thus it was found that the outer cortical layer of the spherical refractive bodies is composed of lipid and protein material, while the inner, medullary portion contains a fluid of unknown composition. The reason for assuming that it is fluid in nature is its eccentric position in the majority of cases. The ratio of the outer lipo-protein layer to the inner portion varies greatly. When the body is larger there is proportionally more of the central fluid present. It seems probable that these bodies grow in size by the increase of the inner fluid portion.

The general staining properties and cytochemical reactions of the spherical refractive bodies are very similar to those of metazoan Golgi substance. The probable mode of increase in size of these bodies, by the progressive accumulation of the central fluid, may be similar to the condensation function of the metazoan Golgi substance. The observations here recorded lend support to the ideas

of previous workers, who claim that the spherical refractive bodies are the Golgi bodies of amebas (MacLennan, 1941).

It has been suggested (Wilber, 1942, 1945b) that the refractive bodies merely represent reserve food or volutin. However, as was first shown by Andresen (1942) and substantiated in the present study, they do not diminish greatly in number under starvation conditions. When they do diminish in number, the remaining bodies become larger, indicating coalescence rather than utilization as reserve food.

The Contractile Vacuoles. The number of contractile vacuoles varies, depending on the species. *Amoeba proteus* and *Amoeba guttula* have only one contractile vacuole. The contractile vacuole of *Amoeba proteus* and related species has undergone a thorough investigation by Mast (1938) and a few other authors, and there is general agreement as to the facts. *Chaos chaos* generally has about 7–10 contractile vacuoles. Each of these vacoules is rather similar in size (80–120 μ before systole) and in behavior to that of *Amoeba proteus.*

Mayorella bigemma has 4–9 contractile vacuoles, with a diameter of about 15 μ before systole. Schaeffer (1918) states that the contractile vacuoles of *Mayorella bigemma* never coalesce. However in the present study of this species it was found that among the smaller, "growing" vacuoles coalescence was fairly common. In the larger vacuoles no coalescence was observed.

There are usually two contractile vacuoles in *Thecamoeba striata.* The outstanding and unique characteristic of this ameba is the curiously shaped larger contractile vacuole. This vacuole is almost never spherical; it is irregularly lobed and constantly changing in shape during locomotion. When the ameba is rounded up and stationary, the contractile vacuole assumes a more regular, rather oval form. Penard (1902) suggested that the curiously lobed appearance of this vacuole is due to the constant formation of smaller new vacuoles adjacent to it, which coalesce with it. During the present study, no evidence was obtained for such an interpretation.

As stated earlier, there are two varieties of *Thecamoeba striata.* In the larger variety, the two contractile vacuoles present include a smaller, spherical vacuole at the posterior and anterior to it, the larger amorphic vacuole which may at times, move forward, carried by the streaming protoplasm. The smaller spherical vacuole sometimes develops into and assumes the position of the larger, lobed vacuole which then disappears. A new spherical vacuole then develops at the posterior end. This, however, is not the general rule. The vacuoles function independently, both emptying their contents to the outside of the cell.

The smaller variety of *T. striata* usually possesses only the amorphic contractile vacuole.

The larger vacuole, in individuals whose length is 80 μ, sometimes reaches a length of 30 μ, but the spherical smaller vacuole is never larger than 15 μ in diameter.

In the past, investigations have ignored or minimzed the possible effects of metabolic activity on the rate of contraction of the contractile vacuole. (See Weatherby, 1941.) Rudzinska and Chambers (1951) report that the pulsation rate of the contractile vacuole in the suctorian *Tokophrya infusionum* accelerated greatly during an increase in metabolic activity of the organism. In *Thecamoeba striata* during locomotion, the time between contractions of the larger lobed vacuole averages 2.5–3 min. The smaller, posterior vacuole takes almost the same length of time. They usually do not contract simultaneously but rather, alternately. In individuals that are not moving but are rounded up and with little or no internal protoplasmic streaming, the time between contractions is about 8 or 10 min.

Howland (1924a) reported that the contractile vacuole in *Thecamoeba* (*Amoeba*) *verrucosa* is not surrounded by beta granules. In *Thecamoeba striata*, however, such a layer of beta granules is definitely present. These granules are not densely packed and occasionally gaps without granules can be found. These granules are

more or less flattened on the surface of the contractile vacuole; upon the contraction of the vacuole, they become spherical. Mast (1938) showed that in *Amoeba proteus* these granules do not determine the site of formation of the new vacuole. In the present study, the same results were obtained with *Thecamoeba striata*. It was also observed, using the phase-difference microscope, that the layer of cytoplasm about 2 μ thick, surrounding the contractile vacuole and containing the beta granules, appears to be in the gel state, and differs in this respect from the cytoplasm surrounding it.

It was found that the beta granules which surround the larger, amorphic vacuole of *Thecamoeba striata* do not in any given period between contractions interchange position with other granules either during the vacuole's rapid changes of shape or during its movement with the streaming protoplasm.

The origin of the contractile vacuoles was investigated in most of the species studied. The evidence indicates that the new vacuole is usually formed *de novo*.

The site of origin can not be predicted by the position of the beta granules that surrounded the old vacuole. However, the new vacuole does form in the approximate position where the earlier vacuole had undergone systole. In *Mayorella bigemma* the formation of a new contractile vacuole is accomplished by the coalescence of at least 6 or 8 very small vacuoles.

It was found on careful observation of the vacuoles of *Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata* that occasionally the vacuole does not contract completely and a minute vacuole, more oval than spherical, measuring 2 to 2.5 μ, remains. The growth of the new vacuole is then merely the enlargement of this minute vacuole. But in a few cases, it was observed that a new vacuole formed adjacent to the minute residual one, and upon subsequent enlargement incorporated the latter.

Crytsals and Crystal Vacuoles. Crystal inclusions were found in *Amoeba proteus*, *Chaos chaos*, and *Mayorella bigemma*. These found in *Amoeba proteus* and *Chaos chaos* are similar and of two types, plate-like and bipyramidal. Their size varies from 2–7 μ and they are always found in vacuoles, which vary in size from 2.5–9 μ. The crystal vacuoles show a great affinity for neutral red, varying from an orange to a light red and deep red color. Some were found to be more alkaline than others, as demonstrated by the color of the neutral red stain. After 24–36 hr., however, most of the vacuoles were stained a deep red.

The ratio of the crystal to the vacuole size varies greatly. Often a small crystal was found in a large vacuole and vice versa. The crystals were usually found to lie eccentrically in the vacuoles. Not more than one crystal was found in any given crystal vacuole.

According to Mast and Doyle (1935a) the plate-like crystals are probably composed of leucine and the bipyramidal ones probably consist of a magnesium salt of a substituted glycine.

Mast and Doyle (1935a) described "blebs" adherent to the surface of some of the crystals, and suggested that these blebs represent the beginnings of the spherical refractive bodies. In the present study such "blebs" were seen only occasionally. It seems very unlikely that such crystals would give rise to the lipid- and protein-composed spherical refractive bodies. Furthermore, it was found that the blebs do not reduce OsO_4, as do the spherical refractive bodies.

In no case did the cytochemical tests indicate the nature of the fluid within the crystal vacuoles. Coalescence of crystal vacuoles was observed only under starvation conditions.

Schaeffer (1918) described in *Mayorella bigemma* small dumb-bell shaped, or hour-glass shaped and occasionally club-shaped crystals as diagnostic for this species. With the use of the polarizing microscope these crystals were easily seen to be anisotropic and strongly birefringent. Schaeffer reported that these crystals have a close affinity to "excretion spheres" which are never larger than 3 μ. Such so-called "excretion spheres" were not found during the present study. These

crystals of *Mayorella bigemma*, unlike those in *Amoeba proteus* and *Chaos chaos*, lie free in the cytoplasm and were not found at any time to be enclosed in vacuoles. These observations were repeatedly checked with vital staining and with the aid of the phase-difference microscope. Their size was 1 to 2 μ through their longest axis.

The Food Vacuoles. The breakdown of food vacuoles into smaller vacuoles was traced in all of the species studied except *Amoeba guttula*. In *Thecamoeba striata* and *Mayorella bigemma* it was found that a newly formed food vacuole very soon (1 to 2 hr.) breaks down into two vacuoles. Within this short time the food organism, *Chlamydomonas*, loses its definite outlines, and the two food vacuoles are colored uniformly green by the presence of chlorophyll. Also desoxyribonucleic acid (DNA), demonstrable by the Feulgen test in the nucleus of the food organism, is found diffused in the resulting two vacuoles. After subsequent breakdown of the secondary food vacuoles into still smaller vacuoles, DNA can only rarely be demonstrated within them, and chlorophyll is no longer present. However, the occasional presence of the DNA might be accounted for in another way which will be discussed later on.

It was not possible in any of the species studied to determine the exact number of times that a newly-formed food vacuole divides into smaller vacuoles. It is probably variable from species to species, and probably to a lesser degree from individual to individual.

In attempting to study the frequency of division of food vacuoles, amebas were placed in a medium containing no food organisms, to avoid confusion arising from the formation of new food vacuoles. It was found that within 24 hr. most of the larger food vacuoles had disappeared and in 36 hr. all of the food vacuoles had disappeared.

During starvation, in those species possessing spherical refractive bodies, some of the latter apparently coalesced. At about this time, numerous non-contractile vacuoles appeared, some of which coalesced. In those species which normally have non-contractile vacuoles (*Chaos chaos* and *Mayorella bigemma*) some of these vacuoles also coalesced and more appeared. Previous investigators (Andresen and Holter, 1945, and Andresen, 1945) who studied the changes in *Chaos chaos* and *Amoeba proteus* during starvation, recorded similar results. The significance of these changes is not understood.

No spherical refractive bodies, beta granules, or crystals were found inside the food vacuoles at any time. This result is supported by observations made earlier on *Chaos chaos* by Andresen and Holter (1942). In contrast, Mast and Doyle (1935a, 1935b) reported crystals and spherical refractive bodies in the food vacuoles of *Amoeba proteus*, and concluded that these inclusions are formed directly by the food vacuole

In all the species studied, small food vacuoles containing undigested food residues accumulate in the posterior end of the ameba and are subsequently egested. It was observed that two or three waste food vacuoles were sometimes egested at one time as if they were one mass, but after their release into the surrounding medium it was found that they had not coalesced but were still separate. The process of egestion was observed in *Amoeba proteus*, *Thecamoeba striata*, and *Mayorella bigemma*.

The Fat Globules. Relatively large numbers of fat globules were found in all of the species of amebas studied. Fat globules were easily seen with the phase-difference microscope; but in the unstained ameba they could not always be differentiated from the smallest spherical refractive bodies, in those species possessing the latter. They measured 1.2–2.5 μ in diameter, being somewhat smaller in the smaller amebas. They colored red with Sudan III and Sudan IV and blackish blue with Sudan black B. The mixture of Doyle (1933) for the simultaneous visualization of starch and fat proved unsatisfactory, since some of the fat globules present in the cytoplasm did not react. The fat globules stained red with Nile

blue sulfate, indicating that they are composed of neutral fats. Negative results were obtained with the Schultz test for cholesterol.

The number and size of the fat globules varied greatly from individual to individual in a given culture. Generally speaking, however, when an ameba appears to be well fed, large numbers of these globules are found, varying in size and scattered throughout most of the cytoplasm. These globules were never within vacuoles of any kind. Under starvation conditions, the number of fat globules decreases. When death from starvation occurs, some fat globules are still present. An estimate of the amount of fat present after death showed that there was great variation from cell to cell. In the majority of the individuals, around one-third of the original total still remained, while in some, very little fat was left. Contrary to the above results, Andresen (1945) found that in *Amoeba proteus* all microscopically visible fat had disappeared by the time death occurred from starvation. However, the present experiments were conducted on *Chaos chaos*, *Thecamoeba striata*, and *Mayorella proteus*, as well as *Amoeba proteus*, and the results obtained were identical in all four species.

Glycogen. Although glycogen was not visible in the untreated cells, it was easily demonstrated in all species studied with the use of Lugol's solution and alcoholic iodine solution. When the amebas had been starved for 36 hr. or more, the results obtained with these iodine solutions were entirely negative.

Permanent vacuoles. Various vacuoles have already been discussed. However, under normal conditions, clear permanent vacuoles are also present in *Chaos chaos* and in *Mayorella bigemma*. These vacuoles differ from contractile vacuoles in that they do not undergo systole and are not surrounded by beta granules. They are readily distinguished from empty crystal vacuoles in *Chaos chaos* because the permanent vacuoles do not show any affinity for neutral red. In *Chaos chaos* permanent vacuoles vary from 2 to 10 μ in diameter. In *Mayorella bigemma* they are usually around 5 μ, although a few have been found as large as 15 μ in diameter. These permanent vacuoles never contain any granules or other particulate matter. Under normal conditions, they never coalesce. During starvation, however, coalescence is common and the size of the vacuoles increases greatly. Also, many new non-contractile vacuoles are formed at this time, and even in *Amoeba proteus* and *Thecamoeba striata* where they are not ordinarily found, similar vacuoles frequently form under starvation conditions.

Neutral red granules. Many observations in this study have been made with the aid of the vital stain neutral red. When amebas are stained with neutral red (Vonwiller, 1913, and many later authors), some of the cytoplasmic inclusions show an affinity for it while others do not. However, certain granules appear which were not visible previous to the neutral red staining. These granules are generally known as neutral red granules. A tremendous amount of confusion concerning them has accumulated in the literature (MacLennon, 1941).

The facts obtained concerning the neutral red granules were about the same in all the species studied. A dilute solution (1:150,000) of neutral red was found to be the most satisfactory for this purpose. About a half hour after this solution was added, numerous small deep red granules about 0.5 to 1 μ in diameter appeared. These granules could not be confused with the alpha and beta granules, which do not take up neutral red. In about 8 hours, more of these deep red granules appeared and some had enlarged to about 2.5 or 3 μ in diameter. Intermediate sizes were also found. In 24 to 48 hr. a few granules measured as much as 6 μ in diameter, and no granules smaller than 1.8 μ were found after a day or two. The color of all these granules, whether large or small was the same deep red. It might be pointed out that the maximum sizes reached by these granules were less in the smaller species than in the larger, being about 4 μ in *Mayorella bigemma* and *Amoeba guttula*.

Extended observations showed no coalescence of neutral red granules in any of the species studied. Up to about 24 hr., the neutral red granules were found free in the cytoplasm. Later, however, these granules were found in vacuoles, usually

one granule, 3 to 6 microns in diameter, per vacuole. The diameter of the vacuoles was found to be 5 to 10 microns. A few vacuoles contained 2 to 3 granules, but these were not common. These intravacuolar neutral red granules accumulate in the posterior portion of the cell during locomotion. Three days after the neutral red solution had been added, the situation had not changed in regard to the number, size, and color of the neutral red granules. However, the number of granules per vacuole had increased from one to as many as 12. The diameter of the vacuoles had increased from 10 to 20 μ, but the number of vacuoles had decreased.

While coalescence of the vacuoles containing neutral red granules was observed a few times, coalescence of the granules themselves was not observed at any time. Therefore the observed increase in size of these granules may be explained as a result of condensation of material but not as the staining of preformed inclusion bodies.

The Cytoplasmic Ground Substance. No exhaustive study was made especially on the cytoplasmic ground substance of the amebas. However, when cytochemical tests were used to determine the distribution of certain compounds in the cell inclusions, it was observed that some of these compounds were found only in the cytoplasmic ground substance or hyaloplasm. Ribonucleic acid (RNA) was found in this ground substance and in the nuclei, but not in any of the cytoplasmic inclusions. Similar results were obtained by Roskin and Ginsburg (1944a, 1944b). Desoxyribonucleic acid (DNA) was demonstrated only in the nucleus and some food vacuoles (Lucas, 1930, and Chalkley, 1936). The results of Chalkley (1937, 1951) showing that sulfhydryl material is found diffuse in the ground substance, were confirmed in the present investigation in *Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*. With the aid of the plasmal reaction, plasmalogen was found diffuse in the ground substance, and not in any cell inclusion. Only the three larger species (*Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*) were tested, as it was not possible to follow the necessary procedure with the smaller amebas. Brachet (1950a) was not able to demonstrate plasmalogens in amebas. However, it is not possible to evaluate these contrary results since he does not tell us what species of amebas he tested or what procedure he followed.

Andresen (1945) and Andresen and Holter (1945) state that during starvation in *Amoeba proteus* and *Chaos chaos* the viscosity of the cytoplasm decreases. Their observations were corroborated in this study. It was found that the heaviest inclusions, the spherical refractive bodies, settled to the bottom of the ameba during starvation, indicating decrease in cytoplasmic viscosity.

DISCUSSION

The criteria for separating genera of the Family Amoebidae are, in general, such gross morphological features as size, number and shape of pseudopodia, number and form of nuclei, etc. (Schaeffer, 1926). The five species here studied represent at least four different genera. They vary in size from 20 microns to 3 millimeters, and the number of nuclei varies from one to several hundred. In these and other respects, these species show great diversity in their gross structure. It is therefore surprising to find a remarkable degree of similarity in their finer structure and cytochemistry. The differences noted are chiefly in the presence or absence of certain components rather than differences in the components themselves. That is, a given cell component shows only slight differences in the various species. This will be brought out in the subsequent discussion.

Cytochemical tests indicate that the outermost layer of all the amebas studied (the "cell membrane"), regardless of thickness, contains neutral mucopolysaccharide material. The tests upon which this finding is based have apparently not been applied to other protozoa. Carbohydrates and proteins in various relationships, are known to be essential constituents of the external membranes of metazoan cells (DeRobertis, *et.al.*, 1948).

Alpha granules (about 0.25 μ in diameter) were found to be present in all the

species studied. Nothing new was ascertained concerning their function, structure, and origin.

Beta granules, predominantly spherical and measuring about one micron in diameter, were found to be present in all the species studied. In the present study, cytochemical methods were applied for the first time to these granules, and confirmed that they are lipid and protein in composition, as are metazon mitochondria. Their staining vitally with Janus green B, as first reported by Mast and Doyle (1935a), was also confirmed. Hence the conclusion of these and other authors that the beta granules represent mitochondria seems to be justified.

It was found with the aid of the phase-contrast microscope that the beta granules are not directly involved in food vacuole digestion, as suggested by Horning and others.

Recent investigations (Bourne, 1950) on liver and other metazon tissues indicate that mitochondria contain a high percentage of those enzymes which play an important part in the aerobic respiratory metabolism of the cell. According to Bourne, many of the enzymes found in mitochondria are constituents of the Krebs tricarboxylic acid cycle, the cycle which has been described as a meeting point of protein, fat, and carbohydrate metabolisms. This indicates that mitochondria may be concerning with breakdown of protein, fat and carbohydrate, as well as with the synthesis of these three substances. Wilber (1945) states that in *Chaos chaos*, after the removal of most of the beta granules, the giant ameba appeared normal. This would seem to indicate that the Krebs cycle enzymes may not be found exclusively in the beta granules, but may be present in the cytoplasmic ground substance as well.

Spherical refractive bodies (present in three of the five species studied) are generally considered to be the Golgi sustance, as first proposed by Brown (1930), because their staining reactions are similar to those of metazoan Golgi material. In general the Golgi elements of metazoan cells are osmiophilic; they stain as a rule with neutral red; and they are believed to act as secretory centers. They are believed to be primarily spheres containing lipid and protein, from which a variety of substances segregate or condense out (Baker, 1944). As already pointed out, the spherical refractive bodies of amebas are always osmiophilic and generally stain vitally with neutral red. The interpretation of Brown would thus seem to be a reasonable one.

It has been shown in the present paper that at least the larger of the spherical refractive bodies in the amebas consist of an outer osmiophilic portion staining with neutral red and an inner portion which shows these reactions very little or not at all. Although the composition of this inner portion was not determined, it might be a condensation substance. It may be further pointed out that the larger sized bodies contain a proportionally greater amount of this inner material, indicating a possible condensation of the outer, corical lipid-protein layer. As in the case of Golgi material of the metazoan cell, the amount of spherical refractive material present in amebas varies from cell to cell and from time to time. It has been shown in this and previous (Andresen and Holter, 1945) studies that the variation in numbers of spherical refractive bodies is not due to their action as reserve food materials, but rather to some other, as yet unknown, physiological condition arising in the ameba. Wilber (1945b) claims that these bodies consist of volutin and contain free aldehydes because they stain with Schiff's reagent without prior treatment. His methods, however, are open to serious criticism. First, the use of Schiff's reagent on cells without prior treatment does not give specific results, and second, the presence in the cell of free aldehydes which have not been unmasked is highly improbable (Hayes, 1949).

Mast and Doyle (1935a) suggested that the origin of the spherical refractive bodies may be directly from the food vacuoles. The present observations do not substantiate such an origin since no spherical refractive bodies were ever found in or

near food vacuoles. Further it seems highly unlikely that both the process of break-down of nutrient material and its resynthesis into cell components should occur in the food vacuole. Extended observations on the food vacuole showed a progressive disintegration of particulate food organisms (digestion), and never a differentiation of this material into anything that would resemble spherical refractive bodies, crystals, or any other type of cell inclusion. Therefore, since there is no definite evidence that these bodies arise from the food vacuoles directly, it may be postulated that they arise from the cytoplasmic ground substance.

Spherical refractive bodies are not present in *Mayorella bigemma* or *Amoeba guttula*, and no other Golgi-like material has been demonstrated in these amebas. This obviously somewhat weakens the interpretation of the refractive bodies as Golgi elements. Another possible difficulty lies in the high refractive index of these bodies; the Golgi material of metazoan cells is notoriously difficult to differentiate from the surrounding cytoplasm. It is of course, conceivable that the function of Golgi bodies may be carried out in some species without the agency of any demonstrable specialized inclusion bodies.

The number of contractile vacuoles present in different species of amebas varies from one to about twelve. However, for a given species the number is fairly constant. It has been found in this study that the rate of pulsation of the contractile vacuole is directly related to the degree of activity of the organism. Similar observations have been made on the contractile vacuole of a suctorian (Rudzinska and Chambers, 1951). It is now generally agreed that the contractile vacuole is primarily a hydrostatic organelle, equalizing the intake and outgo of water. It is conceivable that minute quantities of nitrogenous waste products from the cytoplasmic ground substance may also be excreted (Weatherby, 1941).

Crystals were found in *Amoeba proteus*, *Chaos chaos*, and *Mayorella bigemma*. Those found in *A. proteus* and *C. chaos* were similar and of two types, plate-like and bipyramidal. Their composition was determined by Mast and Doyle (1935a) to be probably leucine and a magnesium salt of a substituted glycine, respectively. These crystals are always found enclosed in vacuoles, which show a great affnity for neutral red. The crystals found in *Mayorella bigemma*, however, are usually dumb-bell shaped and lie free in the cytoplasm, never enclosed in vacuoles.

In a few instances certain "blebs" (first described by Mast and Doyle, 1935a) were observed on the crystals of *Amoeba proteus* and *Chaos chaos*. According to Mast and Doyle these "blebs" represent the beginning of the formation of spherical refractive bodies. To the present writer they appear to be merely imperfections or blemishes on the surface of the crystals. They do not reduce osmium tetroxide as do the refractive bodies.

Mast and Doyle (1935a) postulated that the crystals originate directly from the food vacuoles. No evidence has been found to support this.

In the study of food vacuoles, it was found that the process of digestion is usually accompanied by the division of a vacuole into two or several smaller vacuoles. The undigested residues tend to be massed or packed in the posterior of the organism. Subsequent egestion of these waste vacuoles was observed. These observations approximate those made earlier by Mast (1942).

In a few cases, it was noted that in *Thecamoeba striata* very small food vacuoles (about 3 μ in diameter), which were considered to be waste vacuoles containing food residues, contained desoxyribonucleic acid. It may be postulated that the presence of DNA in these waste vacuoles indicates that at times not all of this compound is broken down during digestion. It is possible, however, that these vacuoles were not waste vacuoles but rather newly formed, small food vacuoles containing bacteria. Ingestion of bacteria alone has not been observed in this species, however.

Egestion of food vacuoles under starvation conditions has been observed in the present study in all the species except *Amoeba guttula*. Andresen (1942) reported this phenomenon in *Chaos chaos*. Mast and Hahnert (1935) state that food

vacuoles in *Amoeba proteus* coalesce during starvation conditions. This was not observed in the present work.

The fat globules were found to be composed of neutral lipids. It was found further that when death from starvation occurred, approximately one-third of the original number of fat globules still remained. The phenomena which occur when amebas are placed under starvation conditions show that starvation does not result in a simple diminution of available food until none exists. The non-availability of food initiates certain phenomena, such as egestion of food vacuoles, vacuolization of the cytoplasm, coalescence and vacuolization of refractive bodies, etc. The significance of these changes is not known. The process of starvation is a very complex one and the end result of that process, death, does not come about simply by the lack of available food. Rather, it probably occurs by the derangement of certain processes, caused by a deficiency of essential compounds.

The permanent vacuoles, normally present only in *Mayorella bigemma* and *Chaos chaos*, are distinguished from contractile vacuoles not only by their non-contractility, but also because they are not surrounded by a layer of beta granules. They are differentiated from empty crystal vacuoles in *Chaos chaos* by the fact that they do not stain with neutral red. These vacuoles normally do not coalesce. Nassonov (1924) thought that the permanent vacuoles together with the contractile vacuoles make up the Golgi complex of protozoa. However, staining properties and behavior of these permanent vacuoles lend no support to this theory. Their origin and function is not known. It is interesting to note that under starvation conditions coalescence and the appearance of new permanent vacuoles occur not only in *Mayorella bigemma* and *Chaos chaos*, where they are normal components, but also in *Amoeba proteus* and *Thecamoeba striata*, where they are not ordinarily found.

On an earlier page we have described the appearance and enlargement of neutral red granules during staining with neutral red, and the subsequent formation of vacuoles around them. Similar observations were made by Andresen (1945). Since the the enlargement of neutral red granules is not brought about by coalescence, and also since these granules have not been demonstrated in the cytoplasm prior to the introduction of neutral red, it is concluded that these granules are not preformed. Probably the action of neutral red on the cytoplasmic ground substance causes certain elements which show a great affinity for the dye to condense or precipitate out and to appear as discrete bodies.

The results obtained in the present study on the cytochemistry of the cytoplasmic ground substance (hyaloplasm) lead to the interpretation that this cytoplasmic ground substance is more than a matrix in which the various inclusion bodies are found. Indeed, it has been suggested that the inclusion bodies to a great degree are merely evidences of the physiological activity of this ground substance.

In metazoan cells (Lazarow, 1943; Claude, 1943; Brachet, 1950) two types of submicroscopic particles have been separated by means of ultracentrifugation from the ground substance: particulate glycogen and the "microsomes." In the present study glycogen was found to be microscopically visible only when treated with an iodine solution. It seems quite possible, therefore, that it is normally present as submicroscopic particles. The action of iodine probably causes these particles to precipitate and to stain reddish brown.

According to Brachet (1950b), the "microsomes" isolated by means of ultracentrifugation were found to contain most of the cytoplasmic RNA, sulfhydrhyl, and plasmalogen. Bayliss (1920) with the aid of the ultramicroscope described certain particles in ameba which are not ordinarily microscopically visible. According to Lazarow (1943) these particles of Bayliss were the equivalent of the "microsomes" which have been subsequently isolated from metazoan cells. However, it is not clear exactly what Bayliss did see as "shimmering points of light." Until all the techniques which have been applied to metazoan cells are adapted and applied to the amebas, no definite statement can be made as to the presence or absence of "microsomes."

SUMMARY

1. A comparative study has been made of the structure and cytochemistry of the cytoplasm of five species in the Family Amoebidae: *Amoeba proteus* (Pallas) Leidy, *Chaos chaos* Linnaeus, *Thecamoeba striata* (Penard) Schaeffer, *Mayorella bigemma* Schaeffer, and *Amoeba guttula* Dujardin.

2. A description is given of the various techniques employed, including the parlodion trap technique, various kinds of staining, and cytochemical methods.

3. The cytoplasmic components studied were: the cell membrane, the alpha and beta granules, the spherical refractive bodies, the contractile vacuoles, the crystals and crystal vacuoles, the food vacuoles, the fat globules, the permanent vacuoles, the neutral red granules, and the cytoplasmic ground substance or hyaloplasm.

4. The five species of amebas studied show great diversity in their size and gross structure. However, on the whole, a remarkable degree of similarity was found in the finer structure and cytochemistry of their cell components.

5. It was found that in all the species studied the outermost layer, or cell membrane, contains neutral mucopolysaccharides. Whether this layer was the seemingly unspecialized plasma membrane of *Mayorella bigemma* and *Amoeba guttula*, or the much thicker, more or less rigid covering (pellicle) of *Thecamoeba striata*, the cytochemical results were the same. The thickness of the pellicle of *Thecamoeba striata* was found to be about 0.33 micron. In both *Amoeba proteus* and *Chaos chaos* the thickness of the plasmalemma is somewhat less, or about 0.25 μ. The cell membrane of *Mayorella bigemma* and *Amoeba guttula* is very much thinner, and it was not possible to measure it.

6. The alpha granules (0.25 μ in diameter) were found to be present in all the species studied.

7. The beta granules in all the species studied are very similar in size (about 1 micron in diameter) and in shape (usually round). In *Thecamoeba striata*, and in no other species studied, coalescence of beta granules was observed. In all cases the beta granules were found to stain with Janus green B. When cytochemical tests were applied to them, it was found that these granules are lipid and protein in composition, as are metazoan mitochondria. No evidence was found that they are involved directly with food vacuole digestion or with the functioning of the contractile vacuole.

8. The spherical refractive bodies were found in only three species (*Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*) of the five studied. They vary in number and size. The larger bodies are not homogeneous, but are divided into an outer cortical layer which stains with neutral red and an inner unstained portion, which is often eccentric in position. These bodies measure from 1.4 to 7 μ in diameter. The ratio of the outer layer to the inner, eccentric portion varies greatly. When the body is larger, there is proportionally more of the central portion present. Cytochemical tests visualize the presence of lipid and protein material in the outer portion. The composition of the inner portion was not ascertained. It seems probable that these bodies represent Golgi material.

9. The number of contractile vacuoles present in amebas is variable. However, for a given species the number is more or less constant. The shape of these vacuoles is almost always spherical. The only exception is the larger of the two vacuoles in *Thecamoeba striata*. This vacuole is irregularly lobed and constantly changing in shape.

10 It has been found in this study that the rate of pulsation of the contractile vacuole is directly related to the metabolic activity of the organism. Further, it was observed that the beta granules surrounding the contractile vacuoles do not determine the site of formation of the new vacuole. It was found that in a few instances the vacuole does not contract completely; a minute vacuole (2–2.5 microns) remains and this is usually the beginning of the next vacuole.

11. Crystal inclusions were found in *Amoeba proteus*, *Chaos chaos*, and *Mayorella bigemma*. Those found in *Amoeba proteus* and *Chaos chaos* are similar, 2 to 7 μ in diameter, and of two types, plate-like and bipyramidal. These crystals are found in small vacuoles (2.5 to 9 μ in diameter). The crystal vacuoles stain readily with neutral red. In a few instances tiny "blebs" were seen on crystal surfaces. It is suggested that these "blebs" do not represent the formation of new refractive bodies as suggested by Mast and Doyle (1935), but rather crystal imperfections.

The crystals of *Mayorella bigemma* are usually dumb-bell shaped and rather small, measuring 1 to 2 μ through their longest axis. Unlike the crystals of *Amoeba proteus* and *Chaos chaos* they are not enclosed in vacuoles, but lie free in the cytoplasm.

12. In the study of food vacuoles, it was found that at least in the four larger species the process of digestion is accompanied by the breakdown of the vacuoles into smaller ones. Egestion of waste food vacuoles was observed. However, under starvation conditions, egestion of any food vacuole may occur.

13. Fat globules were found in all the species studied. They are composed of neutral fats. Usually about one-third of the fat globules are still present when death from starvation occurs. During starvation, all the free glycogen disappears.

14. Permanent non-contractile vacuoles are normally present in *Mayorella bigemma* and *Chaos chaos*. These vacuoles differ from contractile vacuoles in that they do not contract, and are not surrounded by beta granules. They are readily distinguished from empty crystal vacuoles because they do not stain with neutral red. These permanent vacuoles normally do not coalesce, but under starvation conditions coalescence is common, and both size and number of the vacuoles increases. In *Thecamoeba striata* and *Amoeba proteus*, permanent vacuoles appear in the cytoplasm under starvation conditions only.

15. The appearance, color, and growth of neutral red granules have been described, as well as the development of vacuoles around them. Since the growth in size of neutral red granules is not brought about by coalescence, and also since these granules are not found in the cytoplasm prior to the introduction of neutral red, it is concluded that these granules are not preformed. It is thought that they arise through the precipitation of certain material from the cytoplasmic ground substance by the action of the neutral red.

16. Diffused in the cytoplasmic ground substance or hyaloplasm were found ribonucleic acid, plasmalogen, and sulfhydryl. These compounds were not found in any of the cytoplasmic inclusions. The ground substance, it is concluded, is not merely a matrix in which various inclusions are suspended. On the contrary, the inclusion bodies may be looked upon as evidence of the physiological activity of the ground substance itself.

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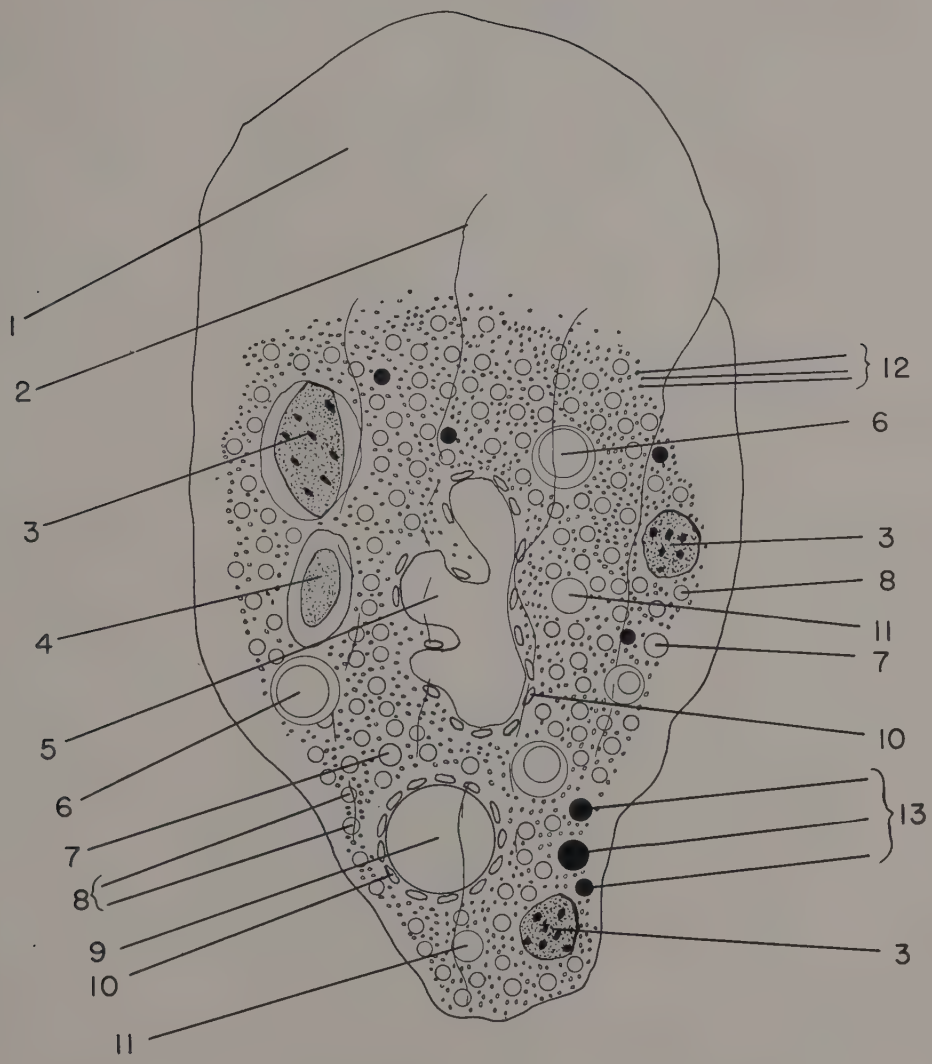
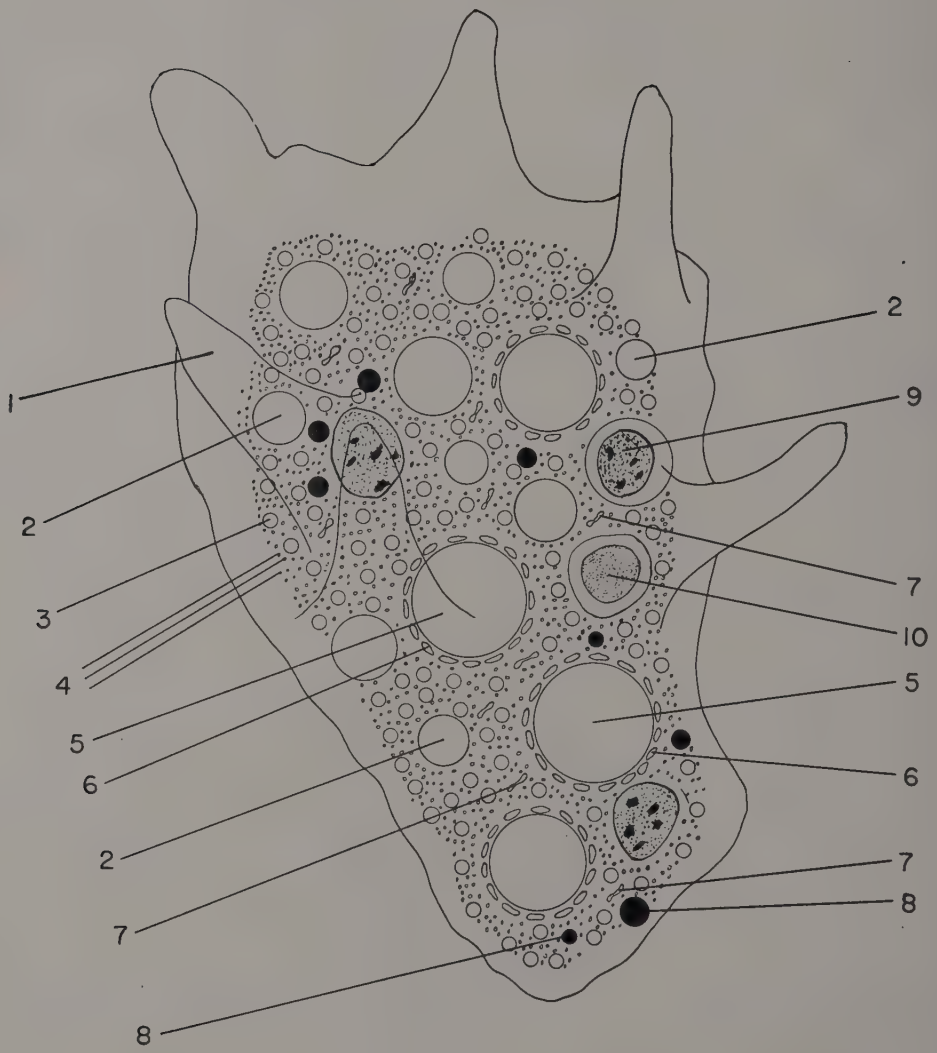


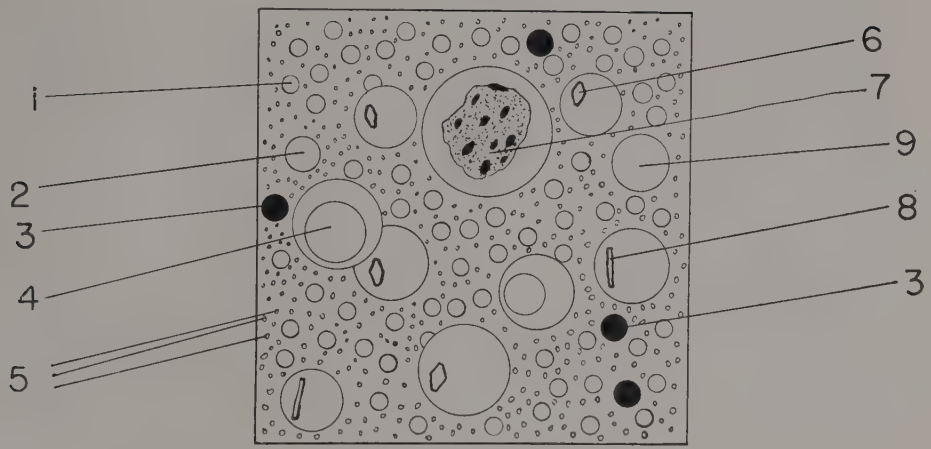
Thecamoeba striata (Penard) Schaeffer. Length, 67.0 μ. Semidiagrammatic.

1—hyaline cap. 2—longitudinal striation or fold. 3—food vacuole. 4—nucleus. 5—amorphic contractile vacuole. 6—larger spherical refractive body. 7—smaller spherical refractive body. 8—beta granules. 9—posterior spherical contractile vacuole. 10—layer of beta granules surrounding the contractile vacuole. 11—coalesced homogeneous sphere of beta granules. 12—alpha granules. 13—fat globules.

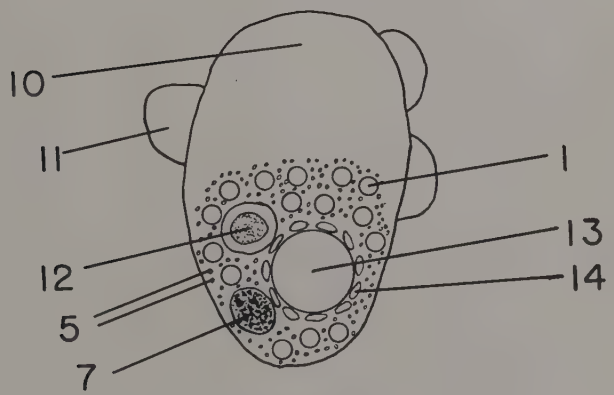


Mayorella bigemma (Schaeffer) Schaeffer. Length, 75.0 μ. Semidiagrammatic.

1—pseudopodium containing hyaloplasm. 2—permanent vacuole. 3—beta granule. 4—alpha granules. 5—contractile vacuole. 6—layer of beta granules surrounding the contractile vacuole. 7—crystal inclusion. 8—fat globules. 9—food vacuole. 10—nucleus.



A



B

A

Diagram showing an area of cytoplasm of *Amoeba proteus* (Pallas) Leidy. Semidiagrammatic

B

Amoeba guttula Dujardin. Length, 20.5 μ. Semidiagrammatic.

1—beta granule. 2—smaller spherical refractive body. 3—fat globule. 4—larger spherical refractive body. 5—alpha granules. 6—bipyramidal crystal in vacuole. 7—food vacuole. 8—plate-like crystal in vacuole. 9—empty crystal vacuole. 10—hyaline cap. 11—pseudopodium containing hyaloplasm. 12—nucleus. 13—contractile vacuole. 14—layer of beta granules surrounding the contractile vacuole.

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Animal Evolution. *G. S. Carter.* Sidgwick and Jackson, Limited, London, and Macmillan Co., New York. 1951. xv+368 pp. $4.50.

In part I the author discusses the biological facts of evolution. A scholarly discrimination of the operation of micro-, macro-, and mega-evolution is presented, and a condensed review of modern genetics supplies the proper background for understanding the factors inherent within the evolving animal. The fine distinctions of biological, systematic and paleontological species reveals the mutability of organisms.

Part II expresses the modern version of the Theory of Evolution which must agree with an amassing wealth of facts. Emphasis on recent views of the causes of evolution strikes an argumentative approach backed by scientific rationalization. An insistence on the simultaneous study of all aspects of the animal's natural history, such as ecology, habits, food, responses, and body organization from the egg to the adult, affecting and effecting the individual and species in all its stages interprets the total environment as the prime mover and underlying cause of evolving organism.

A prodigious bibliography of the most advanced publications on evolution is aptly referred to. This book is highly commendable for a study in the coordination of basic causes of Animal Evolution.

G. J. Siemens.

Organic Chemistry. *H. J. Lucas.* American Book Company, New York. Second edition, 1953. vi+760 pp. $7.00.

The second edition of Professor Lucas' book follows the same philosophy as the earlier edition in its emphasis on fundamental understanding of organic chemistry in terms of a wider physical picture of structure and properties. One of the questions which occur to a teacher in presenting organic chemistry to a beginning class is just how real to the student are concepts such as resonance and electronic mechanisms. The physical reality of these ideas must be emphasized to give them meaning and significance and to make them more than apparent rationalizations. Perhaps the best way to develop this emphasis is to present the basic experimental background of these concepts to the students, as this book does

Complete coverage and the inclusion of useful descriptive organic chemistry has not suffered from the discussion of the physical aspects. The content is such that the text can be used in any one-year beginning course in organic chemistry.

Because of the importance of physical concepts in appreciating and understanding the new knowledge and thought in organic chemistry, I believe this book is to be highly recommended.

EARL W. MALMBERG.

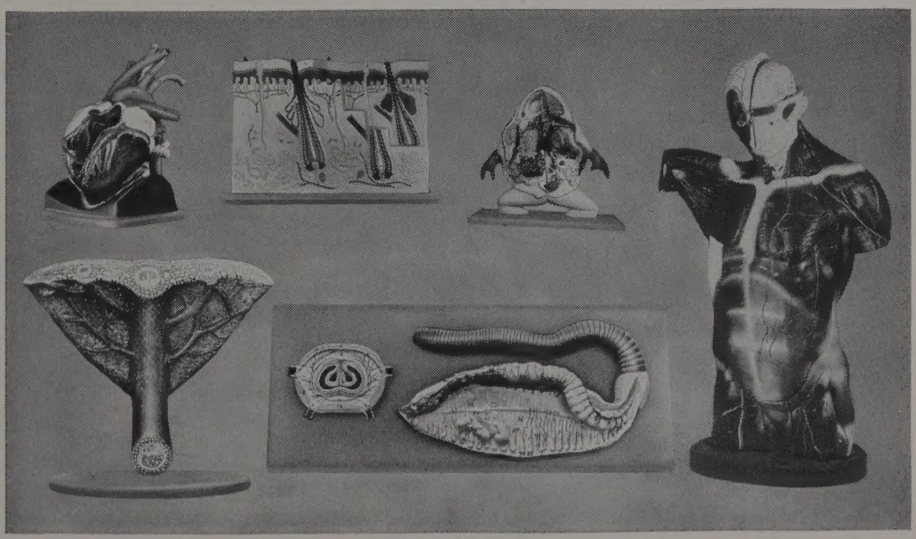
Embryology. *L. G. Barth.* The Dryden Press, Inc., New York 19, N. Y. Revised edition. 1953. xi+516 pp. $6.00.

This book gives an understandable and accurate presentation of the many aspects of embryology. The text is easy to read and the explanations of the embryological phenomena are mostly non-teleological.

The author has made excellent use of illustrations, diagrams, and charts which are so essential to a good embryology text. This book emphasizes the principles of embryology and is so fundamental in many aspects that it would be both useful and valuable as a college text.

One of the excellent features of the book is the compilation of a series of illustrations of the development of the frog, chick and mammal. These pictures are arranged in such a manner that the developmental stages of one animal can be quickly compared with any of the developmental stages of the others.

WILLARD C. MYSER.



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